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SUBSTRATE SPECIFICITIES AND INHIBITION OF INTRACELLULAR ALKALINE PHOSPHATASES ASSOCIATED WITH CYTOPLASMIC GRANULES SEDIMENTED BY DIFFERENTIAL CENTRIFUGATION[1, 2]

FRANCES GREEN KALLMAN[3]
Biology Department, New York University

FIVE FIGURES

Intracellular particles may be divided in general into two groups on the basis of sedimentation differences; the large or microscopic granules, usually considered to include the mitochondria, and the submicroscopic granules including so-called "microsomes." Many investigators have been concerned with determining the properties of such sedimentable fractions but difficulty arises in comparing results since various tissues, various suspending media and various centrifugal forces have been employed. The microscopic granule fraction appears to be the most important site of localization of oxidizing enzymes such as succinic dehydrogenase and cytochrome oxidase as demonstrated in the work of Hogeboom, Claude, and Hotchkiss ('46), Schneider ('46), and Hogeboom, Schneider, and Pallade ('48). Several enzymes have been demonstrated to be associated with the submicroscopic granule fraction such as adenosine triphosphatase in chick embryos (Stein-

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[1] This investigation was supported by a research grant (C-843) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

[2] Taken from a thesis presented to the Faculty of the Graduate School of Arts and Science, New York University, in partial fulfillment of the requirements for the degree of Ph.D. in the Department of Biology.

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JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, VOL. 38, NO. 2
OCTOBER 1951

bach and Moog, '45) and in rat liver (Schneider, '46). The alkaline phosphatase content of a single particulate fraction which probably included elements of both of the fractions mentioned above was studied by Kabat ('41) in mouse kidney homogenate. Later Chantrenne ('47) studied the alkaline phosphatase content of 5 particulate fractions separated from mouse liver homogenate. He found the enzyme activity to be associated with these sedimentable particles though the relative activity of the 5 fractions differed according to whether they were equated in terms of total nitrogen content or ribonucleic acid content.

In the previous studies no attempt was made to study the alkaline phosphatase contained in each fraction in terms of substrate specificity and therefore it was apparent that there was a need for the present study of the alkaline phosphatase content of centrifugally separated cytoplasmic fractions using different phosphate containing substrates and different inhibitors. The tissues used were intestinal mucosa and kidney of the rabbit. In view of the growing interest in phosphatases in respect to protein synthesis and cell metabolism this study of specificity as related to localization seemed to be particularly pertinent.

MATERIALS AND METHODS

Preparation of tissues. In the following experiments kidney and small intestine were removed from rabbits which had been bled by heart puncture and then killed by injecting air into the heart. The intestine was stripped of its contents and washed thoroughly in saline after which both kidneys and intestine were removed to the cold room (0 to 4°C.) where all subsequent preparation of tissue fractions was performed. Kidneys and the mucosa of the small intestine were chosen for this study because of their high alkaline phosphatase activity. The mucosa was scraped from the intestine by splitting it and running a flat blade along the inside surface. The intestinal scrapings were weighed. Each type of tissue was ground in a hand-operated glass homogenizer while being gradually diluted with Homogenizing Solution. In the final dilution 4 ml were added for each gram of tissue. The Homogenizing Solution consisted of 0.042 M KCl, 0.0172 M NaCl, and 0.00043 M $CaCl_2$ calculated by M. J. Kopac (personal communication) to simulate the ionic environment of the cytoplasm. The crude homogenate prepared in this procedure was subsequently submitted to a modification of the centrifugal fractionation procedure outlined by Claude ('46).

Tissue fractionation. All centrifugation was performed in the cold room. Nuclei, unbroken cells and tissue debris were first removed from the crude homogenate by three separate centrifugations at 1,300 g using a Clay Adams angle head centrifuge. The residue from each was discarded. The final supernate or Refined Homogenate consisted of a suspension of cytoplasmic particles from which the particulate fractions were separated. Figure 1 represents the centrifugation procedure followed in the preparation of the Refined Homogenate.

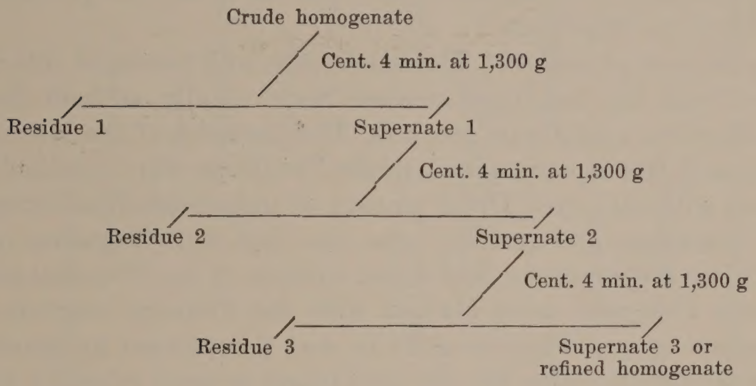

Fig. 1 Centrifugation procedure followed in the preparation of the Refined Homogenate.

A large particles fraction, the Microscopic Granule Fraction, consisting largely of particles which sedimented after 40 minutes' centrifugation at 1,300 g was separated from a small particles fraction, the Submicroscopic Granule Fraction, consisting of a pellet which formed as the result of submitting the supernate of the previous centrifugation, minus Residue 6, to 90 minutes' centrifugation at 18,000 g. Figure 2 represents the centrifugation procedure followed in the preparation of these fractions. A motor-driven, self-balancing, angle type centrifuge built by Blum of Rockefeller Institute for Medical Research and operated *in vacuo* was used for centrifugation at 18,000 g. The final fractions which were tested for phosphatase activity included samples of the Refined Homogenate, the Microscopic Granule Fraction, the Submicroscopic Granule Fraction, and the relatively granule-free supernate resulting after the removal of the last particulate fraction, Supernate 7.

Cytological procedure. The whole cells and pieces of intact tissue from the intestinal mucosa were vitally stained for mitochondria with Janus green B. The particles of the Microscopic and Submicroscopic Granule Fractions were similarly treated with this dye. Dried smears of unhomogenized intestinal scrapings and residue from the first centrifugation of the Crude Homogenate, and dried smears of the Microscopic Granule Fractions were stained with the Feulgen reaction.[4]

Enzyme assay. Enzyme activity was determined by measuring colorimetrically for the phosphate groups released by the enzyme according to the method of Soyenkoff ('47). The method of Miller and Miller ('48) was used for nitrogen determinations which were performed on the fractions in order to equalize them in terms of total protein content. Appropriate dilutions of each of the centrifugal fractions in $2\frac{1}{2}$ ml quantities were incubated at 37°C. with an equal volume of substrate solution or substrate-inhibitor solution. Samples of 1 ml were removed from the enzyme-substrate mixture at 0, 15 and 30 minutes and the enzyme reaction was stopped immediately by placing the sample in 9 ml of 10% trichloracetic acid. Phosphate determinations were performed on each of these samples removed and enzyme activity was expressed in moles of phosphate released per milligram nitrogen within a certain time.

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[4] The author wishes to thank Dr. Milton A. Lessler for performing this technique.

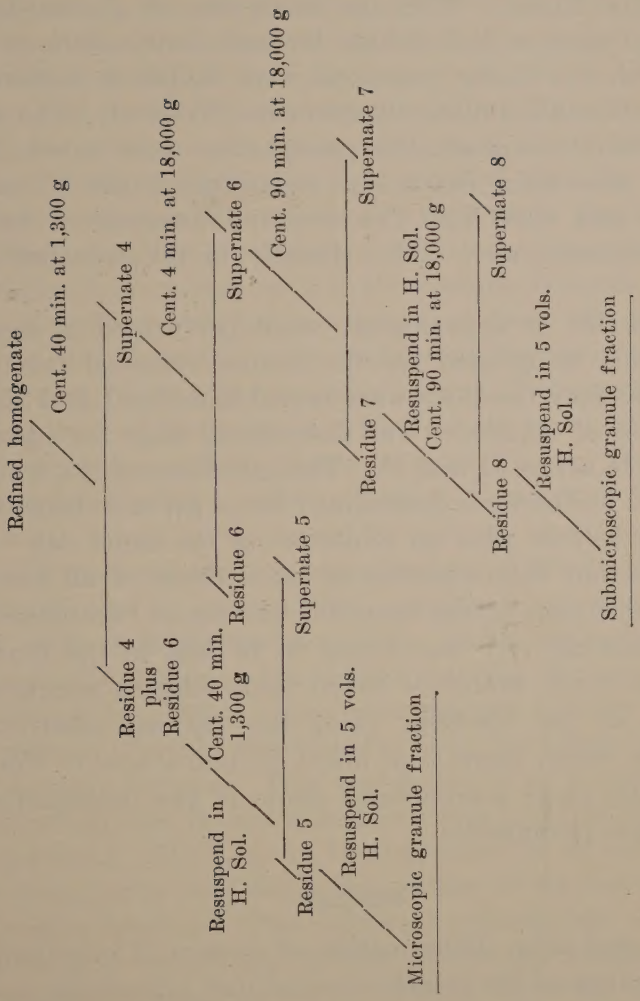

Fig. 2 Centrifugation procedure followed in the preparation of the particulate fractions.

The substrates used included beta-glycerophosphate sodium salt, alpha-glycerophosphate calcium salt, alpha-beta-glycerophosphate (Eastman Kodak Co. mixture) sodium salt, fructose diphosphate magnesium salt, adenosine-3-phosphoric acid, glucose-1-phosphate di-potassium salt, glucose-6-phosphate barium salt, and yeast nucleic acid sodium salt (courtesy Dr. Martin Kuna). With the exception of glucose-1-phosphate and glucose-6-phosphate the substrate solutions incubated with the tissue fractions were 0.0127 M buffered to pH 9.4 with sodium diethylbarbiturate (Veronal). The action of three inhibitors of alkaline phosphatase were tested. These included phlorhizin (0.001 M), *1*-histidine (0.064 M) and *1*-glutamic acid (0.03 M). The required amounts of each of these substances were added directly to the substrate solutions.

Four series of experiments were performed a separate series being designated for the tissues removed from each animal. Kidney fractions were tested in series I and II only. The action of inhibitors was determined only for intestinal fractions in series II and IV. The activities of the fractions of a given series were determined for a given substrate and for the substrate plus an inhibitor on the same day. Time did not permit determination of the activity of all fractions with all substrates on the same day or even on two consecutive days. Since activity was found to be lost in the fractions with time it was therefore found impossible to compare the activities of the fractions using the various substrates if they were tested more than about 28 hours apart. For this reason only those comparisons made in the presentation of the data are permissible.

RESULTS

Cytological data. Examination of smears of intestinal mucosa scrapings of the rabbit revealed that mitochondria could be seen within the cells appearing as small round to rod-shaped granules in the cytoplasm. These could be observed with a compound microscope after staining with Janus green B and with the phase contrast microscope in the absence of staining. Smears of the Microscopic Granule Fraction of the intestinal mucosa stained with Janus green B revealed that numerous small granules were present in this preparation and that no particles above about 2 μ in diameter were present. The detection of color in these preparations was extremely difficult although it was estimated that a majority of the granules were stained with Janus green B. No particles could be observed in the Submicroscopic Granule Fraction.

Smears of whole cells taken from scrapings of mucosa and treated by the Feulgen reaction revealed that the nuclei of the intact mucosa cells stained darkly while the cytoplasm remained considerably lighter. No stained particles could be observed in smears of the Microscopic Granule Fraction treated similarly. In smears prepared from the residue of the first centrifugation many nuclei could be observed to stain darkly with the Feulgen reaction. Many of these nuclei were floating free in the medium while others could be observed within unbroken cells in pieces of intact tissue. The isolated nuclei were similar in size and shape to many of those seen within the intact cells. No nuclei or large particles were observable upon microscopic examination of the residues.

Distribution of phosphatase activity in particulate fractions with beta-glycerophosphate. With beta-glycerophosphate as the substrate the Submicroscopic Granule Fraction was found to exhibit more phosphatase activity than the Microscopic Granule Fraction for both the kidney and intestinal mucosa material. This was tested in all 4 series for the intestinal mucosa fractions and in series I and II for the kidney fractions. The Refined Homogenates in all series tested were found to be less active than either of the corresponding particulate fractions. The relatively granule-free supernates, Supernates 7, were found to have practically negligible activity. Figure 3 represents curves of the 4 fractions of the intestinal mucosa homogenate in series IV. Similar results were obtained for each type of tissue tested for each series.

Distribution of phosphatase activity in particulate fractions with fructose diphosphate. In series I and II the relative activity of the fractions of the intestinal mucosa and kidney homogenates were tested with the substrate fructose diphosphate. In both series it was found that the Microscopic Granule Fractions of the kidney and intestinal mucosa were more active than the corresponding Submicroscopic Granule Fractions. This was the opposite of the distribution found with the substrate beta-glycerophosphate. Figure 4 represents curves of phosphatase activity of fractions of intestinal mucosa and kidney homogenates in series I with fructose diphosphate. Figure 5 represents curves of phosphatase activity of the same fractions with beta-glycerophosphate as the substrate.

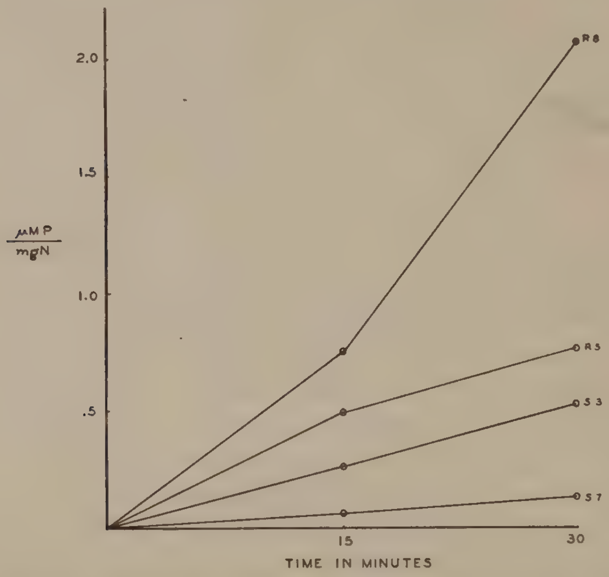

Fig. 3 Alkaline phosphatase activity with beta-glycerophosphate showing the relative activities of 4 centrifugal fractions of intestinal mucosa homogenates. R8 Submicroscopic Granule Fraction, R5 Microscopic Fraction, S3 Refined Homogenate, S7 Granule-free supernate.

Relative phosphatase activity of the Microscopic and Submicroscopic Granule Fractions with other substrates. The relative activities of the Microscopic and Submicroscopic Granule Fractions of intestinal mucosa in series II and IV were tested with 6 substrates other than those previously mentioned. It was found that the Submicroscopic Granule Fraction had more activity than the Microscopic Granule Fraction with alpha-glycerophosphate, alpha-beta-glycerophosphate (Eastman Kodak mixture), glucose-1-phosphate, glucose-6-phosphate, adenosine-3-phosphoric acid, and yeast nucleic acid. The data presented are in tabular form according to which substrates were tested sufficiently close together in time to be comparable.

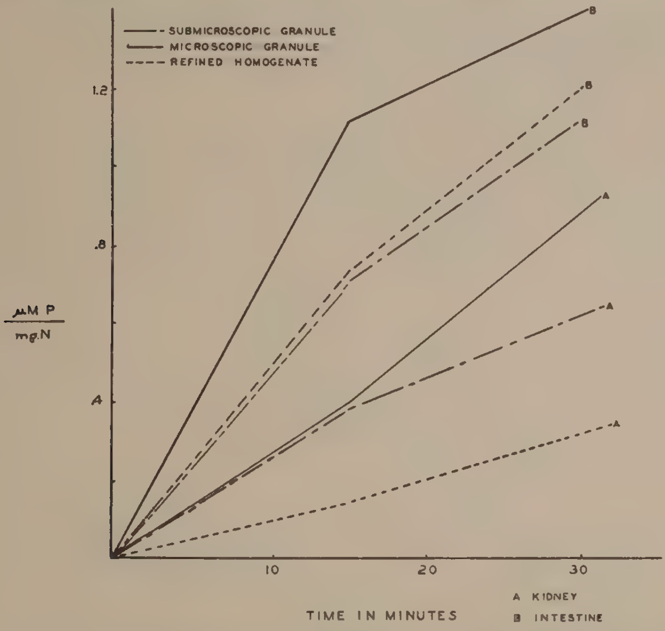

Fig. 4 Alkaline phosphatase activity with frucose diphosphate demonstrating the relative activities of three particulate fractions separated from kidney and intestinal mucosa homogenates by centrifugation.

In table 1 are presented the relative activities of the Microscopic and Submicroscopic Granule Fractions of intestinal mucosa of series II and IV with beta-glycerophosphate, alpha-glycerophosphate and a mixture of these two substrates. Activities in moles of phosphate released per milligram of nitrogen are presented for 15 to 30 minutes' incubation. From examination of the data it becomes apparent that the overall activities of both particulate fractions are greater with alpha-glycerophosphate than with beta-glycerophosphate. The two fractions of any one series exhibited about the same proportional increase in activity when alpha-glycerophosphate was used instead of beta-glycerophosphate.

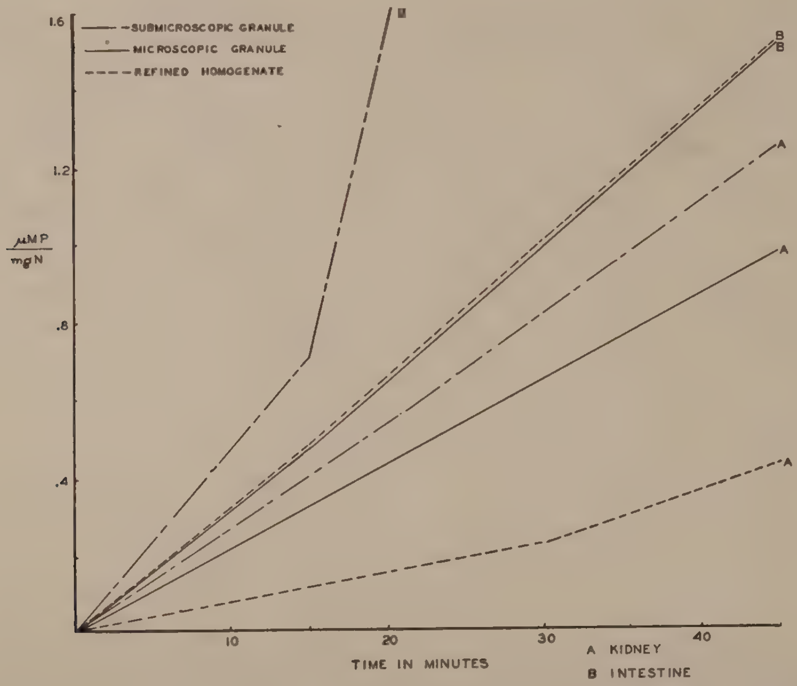

Fig. 5 Alkaline phosphatase activity with beta-glycerophosphate demonstrating the relative activities of three particulate fractions separated from kidney and intestinal mucosa homogenates by centrifugation.

The data for the relative activities of the Microscopic and Submicroscopic Granule Fractions of intestinal mucosa of series II and IV with glucose-1-phosphate and glucose-6-

phosphate are presented in table 2. In both series the two particulate fractions demonstrated more activity with glucose-1-phosphate than with glucose-6-phosphate. The proportional increase was approximately the same for both particulate fractions when glucose-1-phosphate was substituted for glucose-6-phosphate.

With two other comparable substrates, adenosine-3-phosphoric acid and yeast nucleic acid, it was found that the activity of both fractions of series II and IV was greater with the first named substrate than it was with the last. In table 3 the data are presented which represent the relative activity of the two fractions with these two substrates. The increase in phosphatase activity was proportional in both fractions when the substrate was changed from the less active to the more active substrate.

TABLE 1

Phosphatase activity expressed in μM of phosphate released per milligram of nitrogen after 15 and 30 minutes' incubation at 37°C.

		BETA-GLYCEROPHOSPHATE		ALPHA-GLYCEROPHOSPHATE		ALPHA-BETA-GLYCEROPHOSPHATE	
Series		II	IV	II	IV	II	IV
R5	15 min.	1.82	0.29	1.93	1.15	*	0.27
	30 min.	1.91	0.65	2.99	1.27	*	0.67
R8	15 min.	2.44	0.38	2.96	2.27	*	0.57
	30 min.	4.20	1.84	4.98	4.97	*	2.20

R5 Microscopic granule fraction.
R8 Submicroscopic granule fraction.

Effect of time on phosphatase activity. For each series of experiments the work was necessarily spread over a number of days. For this reason it became important to determine what effect time had on the phosphatase activity in the fractions. Accordingly experiments were performed in series II to determine what effect time had on activity of fractions with respect to one or more substrates. A measurable drop in activity was found with both fractions when activity was measured before and after a 6-day period with beta-glycerophosphate as the substrate and before and after a 10-day period with alpha-glycerophosphate. Table 4 represents the per cent of activity remaining in the intestinal mucosa fractions of series II when tested with the two substrates after the times specified. From this it will be seen that in both fractions a larger per cent of the original activity remains with alpha-glycerophosphate than with beta-glycerophosphate in spite of the fact that the time lapse was greater with alpha-glycerophosphate than with beta-glycerophosphate.

TABLE 2

Phosphatase activity expressed in μM of phosphate released per milligram of nitrogen after 15 and 30 minutes' incubation at 37°C.

		GLUCOSE-1-PHOSPHATE		GLUCOSE-6-PHOSPHATE	
Series		II	IV	II	IV
R5	15 min.	3.06	0.31	1.17	0.19
	30 min.	4.91	0.43	1.61	0.32
R8	15 min.	4.62	0.82	1.28	0.49
	30 min.	5.86	1.46	1.86	0.83

R5 Microscopic granule fraction.
R8 Submicroscopic granule fraction.

Effects of inhibitors of phosphatase activity. In order to elaborate the correlation of substrate specificity with the two particulate fractions of centrifuged homogenates from intestinal mucosa preliminary experiments were performed in which three inhibitors, *1*-histidine, *1*-glutamic acid and phlorhizin, were tested with each fraction for each substrate. Only those findings are presented which illustrate differences using structurally related compounds with the two particulate fractions.

The results show that phlorhizin caused greater inhibition in both fractions when alpha-glycerophosphate was used than when beta-glycerophosphate was used. Also *l*-glutamic acid in general inhibited both fractions more with alpha-glycerophosphate than with beta-glycerophosphate. The data for the action of the inhibitors with the substrate alpha-beta-glycerophosphate are not identical with those in which either of the other two glycerophosphates were used alone.

TABLE 3

Phosphatase activity expressed in µM of phosphate released per milligram of nitrogen after 15 and 30 minutes' incubation at 37°C.

		ADENOSINE-3-PHOSPHORIC ACID		YEAST NUCLEIC ACID	
Series		II	IV	II	IV
R5	15 min.	*	0.75	0.83	0.13
	30 min.	2.42	1.22	2.00	0.29
R8	15 min.	1.71	1.33	1.20	0.37
	30 min.	3.01	1.98	2.33	0.59

R5 Microscopic granule fraction.
R8 Submicroscopic granule fraction.

Both the Microscopic and Submicroscopic Granule Fractions are inhibited more by phlorhizin, *l*-histidine and *l*-glutamic acid when glucose-6-phosphate is used than when glucose-1-phosphate is the substrate.

DISCUSSION

The cytological appearance of the fractions afforded a rough indication of the nature of the particles separated by centrifugation. The fact that the particles in the intact cell and those in the Microscopic Granule Fraction stained similarly with Janus green B indicates that the particles observed in the intact cell were mitochondria and that those seen in the fraction may be mitochondria or at least may be derived from them. The ability of isolated particles to stain with Janus green B is a good though not absolute criterion by which to judge their identity with mitochondria. The fact that no visible particles were present in the Submicroscopic Granule Fraction supports the assumption that this fraction consists of particles of submicroscopic dimensions.

The integrity of the fractions is demonstrated by the fact that nuclei are found to sediment intact in the preparations. Proof that the large sedimented round structures are nuclei is offered by the fact that they are Feulgen-positive. Since no Feulgen-positive material was found in the particulate fractions it can be assumed that probably no nuclear materials contaminate these cytoplasmic fractions.

TABLE 4

Values represent per cent of phosphatase activity remaining in two particulate fractions of series II after loss of activity with time

	R5 MICROSCOPIC GRANULE FRACTION	R8 SUBMICROSCOPIC GRANULE FRACTION
Alpha-glycerophosphate (After 10 day period)	15 min. reading 77%	15 min. reading 83%
	30 min. reading 70%	30 min. reading 87%
Beta-glycerophosphate (After 6 day period)	15 min. reading 66%	15 min. reading 79%
	30 min. reading 63%	30 min. reading 78%

In the methods used for the removal of tissues and preparation of homogenates every precaution was taken to disturb the enzyme and the cell as little as possible. The solution into which the cells were broken was chosen in order to reproduce the ionic strength of the cytoplasm as closely as can be calculated from present evidence. It differs considerably from the 0.85% sodium chloride solution or the 1 N sucrose solution more commonly used as a disintegrating medium and it contains potassium chloride which Claude ('41) states causes clumping and precipitation of granules. In this connection it must be remembered that a solution of mixed salts such as

Homogenizing Solution has very different properties from a solution containing a single salt such as potassium chloride. Thus, while other investigators have used hypertonic sucrose (Hogeboom, Schneider and Pallade, '48) and buffered saline (Claude, '41; Chantrenne, '48), the present medium appears to have afforded good separation of granules from intestinal mucosa cells and is more nearly similar to what we know must exist as the ionic strength of the cytoplasm. Other investigators have worked exclusively with different cells which may account in part for differences in results.

In the present experiments the results obtained with beta-glycerophosphate agree with the findings reported by Kabat ('41) and Chantrenne ('47) in that the phosphatase activity of the refined kidney and intestinal mucosa homogenates is found to be associated with sedimentable particles. From the data here presented it is evident that after fractionation little phosphatase activity remains in the Supernate 7 after removal of the granular fractions. This does not exclude the possibility, however, that the Supernate 7 may contain particles of a size which do not sediment at the centrifugal forces used and which may also contain limited amounts of phosphatase activity. As indicated by the experiments of Chantrenne ('47), it seems likely that the cytoplasm contains particles of varying sizes which sediment over a wide range of centrifugal speeds. Judging from this evidence it is probable that the fractions separated in the present experiments contain particles of varying sizes within a given range.

The problem of specificity of cytoplasmic enzymes associated with particles appears to offer potentialities in relating knowledge of cytoplasmic structure with function and may help elucidate such questions as the role of the plasmagene and the virus in life processes.

The results of the present study indicate that there are probably two separate phosphatases which hydrolyse beta-glycerophosphate and fructose diphosphate and which are differently distributed with respect to the cytoplasmic granules. The fact that there is consistently more activity in the

Microscopic Granule Fraction than in the Submicroscopic Granule Fraction with fructose diphosphate suggests that this enzyme is differently distributed with respect to the cytoplasmic granules than is the phosphatase which hydrolyses beta-glycerophosphate. The existence of a separate diphosphatase was predicted by Kay ('32) and later Gomori ('43) demonstrated that the enzyme which hydrolysed fructose diphosphate had different properties from that which hydrolysed glycerophosphate. Differences in distribution with respect to cytoplasmic granules have not, however, been noted previously.

For many years the phosphomonoesterases have been considered to be non-specific enzymes since most preparations hydrolysed a number of substrates. Recently, however, the specificity of these enzymes has been more critically examined and some evidence of their specificity arises from histochemical studies in which differences in distribution result after using different phosphate containing substrates. Until it is possible to prepare purified phosphatases it will be impossible to obtain absolute confirmation of this from studies of isolated cytoplasmic fractions. The best that can be hoped for is cumulative evidence from studies such as the one here reported.

The data indicate that the particulate fractions evidence more phosphatase activity when alpha-glycerophosphate is the substrate than when beta-glycerophosphate is used. With both substrates, however, the Submicroscopic Granule Fraction had more activity than the Microscopic Granule Fraction. This observation may be interpreted to indicate that some difference in the hydrolysis of these two substrates is present although it does not necessarily indicate that a different enzyme hydrolyses each substrate. Evidence that alpha-glycerophosphate and beta-glycerophosphate are hydrolysed at different rates by phosphatases of both plants and animals is contributed by Kay ('26).

Although the Submicroscopic Granule Fraction remains the most active fraction when alpha-glycerophosphate is used as a substrate instead of the beta-derivative, the relative increase in this fraction over the Microscopic Granule Fraction is perhaps somewhat greater. It is possible, therefore, that there are separate enzymes which hydrolyse both substrates in both fractions but that the enzyme which hydrolyses alpha-glycerophosphate is relatively more concentrated than the enzyme which hydrolyses beta-glycerophosphate in the Submicroscopic Granule Fraction. On the other hand, a phosphorylating enzyme such as described by Meyerhoff and Green ('49) may cause a greater synthesis of glycerophosphate in the presence of one substrate or one fraction and thus reduce the amount of measurable phosphatase. This might account for the apparent differences in phosphatase activity when the two glycerophosphates are used with the two particulate fractions.

When the activities of two particulate fractions were studied using glucose-1-phosphate and glucose-6-phosphate it was found that the phosphatase which hydrolysed the former was more active than the phosphatase which hydrolysed the latter. These data suggest that conversion of glucose-1-phosphate to glucose-6-phosphate may not occur in these fractions. If such a conversion did occur, as was found by Broh-Kahn and Mirsky ('49), one would expect the phosphatase activity of any one fraction to be the same with both glucose-1-phosphate and glucose-6-phosphate. Since striking differences are apparent using these two substrates, it appears that if phosphoglucomutase is present it is not sufficiently concentrated to convert a large percentage of glucose-1-phosphate to glucose-6-phosphate. Analysis of the data presented indicates that there may be one phosphatase which hydrolyses glucose-1-phosphate which is distinct in its properties and perhaps its distribution from the enzyme which hydrolyses glucose-6-phosphate. Interpretation of these data, however, is subject to the same limitations as are discussed with relation to the glycerophosphates.

The inhibitors used in the present study are commonly known inhibitors of alkaline phosphatase. Phlorhizin was established quite early as an inhibitor. Amino acids, including *l*-histidine and *l*-glutamic acid, are inhibitors of alakline phosphatase above a minimal concentration (Bodansky, '48). The strongest indication lent by the data from the use of inhibitors was that two different enzymes may hydrolyse alpha-glycerophosphate and beta-glycerophosphate and that they may be distributed in relatively the same proportion in both of the particulate fractions. This may be inferred from the fact that the three inhibitors are found to have greater inhibitory action on the phosphatase which hydrolyses alpha-glycerophosphate although it may also be interpreted as indicating that the three inhibitory substances interact differently with the two substrates and therefore interfere differently with the action of a single enzyme which hydrolyses both substrates.

In considering the overall picture of the problem attacked and the results obtained it appears that some indications of specificity between two particulate fractions are evident when different phosphate containing substrates are used. It is likely that sharper centrifugal separation of cytoplasmic particles may indicate even greater specificities particularly between beta-glycerophosphate and fructose diphosphate. Maximal maintenance of the native properites of the isolated cytoplasmic constituents will contribute to the validity of any such future studies.

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SUMMARY

Alkaline phosphatase activity was determined colorimetrically in particulate fractions separated by differential centrifugation according to a modification of the method of Claude ('46) from homogenates of rabbit kidney and intestinal mucosa. Two particulate fractions were prepared; a Microscopic Granule Fraction consisting of particles which

sedimented from a refined homogenate after 40 minutes' centrifugation at 1,300 g and a Submicroscopic Granule Fraction which separated after 90 minutes' centrifugation at 18,000 g. The kidney fractions were found to have less activity than the intestinal mucosa fractions. No qualitative differences were noted between the fractions of the two types of tissue. With the substrates beta-glycerophosphate, alpha-glycerophosphate, alph-beta-glycerophosphate, glucose-1-phosphate, glucose-6-phosphate, adenosine-3-phosphoric acid, and yeast nucleic acid, the Submicroscopic Granule Fraction of the intestinal mucosa had more activity than the Microscopic Granule Fraction. With the substrate fructose diphosphate the Microscopic Granule Fraction had more activity than the Submicroscopic Granule Fraction. This was interpreted as indicating that the phosphatase which acts on this substrate is differently distributed with respect to the particulate fractions than is the enzyme which acts on the other substrates.

No other conclusive interpretation could be drawn from the data although it was indicated that the enzyme which hydrolyses alpha-glycerophosphate may be different from the enzyme which hydrolyses beta-glycerophosphate and that the phosphatase which hydrolyses glucose-1-phosphate may be different from the enzyme which hydrolyses glucose-6-phosphate.

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FILTRATION RATE AND WATER DIURESIS IN THE SEAL, PHOCA VITULINA

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TWO FIGURES

The theory that water reabsorption in the renal tubules is partitioned between an obligatory (proximal) and a facultative (distal) process (Smith, '51) is based in part on the fact that of the total water contained in the glomerular filtrate only a limited fraction (on the average about 15%) is available for excretion during water diuresis. Ludemann, Raisz, and Wirz (personal communication) have shown in the dog that this fraction varies within the range of 11 to 19% when the filtration rate is raised or lowered by changing the protein content of the diet. In the marine seal, *Phoca vitulina*, the filtration rate may be increased markedly by feeding (Hiatt and Hiatt, '42) and greatly reduced by excitation of the diving reflex (Bradley and Bing, '42). This animal therefore affords an excellent opportunity to examine the influence of wide changes in filtration rate on facultative water excretion.

METHODS

The animals used in this study consisted of 15 female and 5 male young seals captured shortly after weaning and weighing 16 to 26 kg, and one 1-year-old female weighing 31 kg.[3]

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They were kept in floating cages and trained to eat fresh herring from a tub immersed at the bottom of the cage. The majority received frequent intramuscular injections of penicillin as a prophylactic and therapeutic measure against infection, which they readily developed at the site of venepuncture.

In order to induce water diuresis it was necessary to accustom the animals to the introduction of a stomach tube for administration of water, for which purpose they were repeatedly given small water loads (500 cm^3) before definitive studies were begun.[4] During the experiment the animal was strapped on a V-shaped animal board and given 50 to 150 cm^3 of water per kilogram, either as a constant drip or in small doses. Urine was collected from females at 2 to 30 minute intervals through an inlying bladder catheter with manual compression of the abdomen. The males, which could not be catheterized, were placed prone on the animal board where they would void into a funnel at intervals which varied with the urine flow. Blood was drawn at appropriate intervals from a vein in one of the flippers, usually by means of an indwelling no. 21 needle.

The filtration rate was measured by the exogenous creatinine clearance (Smith, '36), for which purpose creatinine was given orally in doses of 3 to 5 gm at the time of water administration. The filtration rate of water is taken as wC_F, where w is 1 — (protein content in gm/cm^3) and C_F is the creatinine clearance in cm^3/min. Creatinine was determined by the Bonsnes-Taussky ('45) method in urine and in a 1:15 sodium tungstate filtrate of plasma, plasma chlorides by iodometric titration (Van Slyke and Hiller, '47), urea by the hypobromite method of Van Slyke and Kugler ('33), plasma protein by the copper sulphate method of Van Slyke et al.

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[4] In order to avoid the time-consuming process of training, sedation was attempted in a few experiments; however, nembutal, alcohol and chloralose all inhibited water diuresis and reduced the filtraton rate.

('50) and the freezing point of plasma and urine by the Johlin ('31) method, using a Beckmann thermometer.

Since the animals differed considerably in size, data on urine flow and filtration rate have been corrected to 100 gm of kidney weight, with the assumption that kidney weight varied in proportion to body weight during the summer. The relation that KW in gm = 9.6 BW in kg was determined from kidneys which were cleaned but not decapsulated, and represents 9 animals in fair nutritional state.[5]

Maximal values for the filtration rate were obtained 2 to 4 hours after the forced feeding of 1 kg or more of fresh herring, moderately low values by fasting the animals for 18 hours, and minimal values during the diving reflex which is elicited by closing the nostrils by means of a cone placed over the snout. It was assumed that this reflex was activated when the heart rate dropped rapidly to the range of 10 to 20 beats per minute. The possible effects of accomodation to any procedure were minimalized by alternating experiments at low and high filtration rates. Efforts to increase the filtration rate by the intravenous administration of albumin and amino acids were unsuccessful.

RESULTS

For a variety of reasons we were successful in obtaining maximal water diuresis in only 43 out of 105 experiments on 21 seals.[6] These successful experiments were carried out on 12 animals. In these experiments the filtration rate ranged from 6.8 cm^3/min. during the diving reflex to 132 cm^3/min. per 100 gm kidney during the absorption of the fish meal. The minimal creatinine U/P ratio during maximal water diuresis at these extreme levels of filtration rate ranged from 7.9 to 2.0, representing the excretion of 12.7 to 3.7% of filtered water.

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[5] Bradley and his co-workers (personal communication) have obtained a factor of 8.4 from 11 animals weighing 13.8 to 30.2 kg.

[6] It is not surprising that fright, struggle, etc., should frequently elicit antidiuresis even in the face of a heavy water load in these animals. Antidiuresis was generally accompanied by a reduction in the filtration rate, oliguria, unconsciousness, hyper-reflexia, salivation, and tearing, and terminated in respiratory failure. Such animals sometimes seemed to be revived by artificial respiration and the intravenous administration of hypertonic saline solution, and later showed good diuretic responses. However, 8 animals died of what was presumably water intoxication.

Although there is wide scatter in the data, the urine flow during maximal water diuresis in any one animal increases in a fairly uniform manner with increasing filtration rate, a relation also evident from the massed data presented in figure 1, where the urine flow as per cent of filtered water ($V/C_F \times 100$) is plotted against the filtration rate of water per 100 gm of kidney. Deviations from the average trend could not be correlated with other variables such as chloride excretion, plasma chloride concentration, plasma urea concentration or the magnitude of the water load. Because of the limited number of observations on any one animal we have not analyzed the data on individual animals, but we have calculated the linear regression line for the massed data. The correlation coefficient is + 0.7173, and the intercept on the ordinate axis is 5.2% at a filtration rate of zero. The mean filtration rate of the entire series is 50 cm^3/min. per 100 gm kidney, and the mean value of $V/C_F \times 100$ is 8.2%.

Because renal function in the seal is so labile and related to the assimilation of food, it is impossible to designate any filtration rate as physiologically comparable to standard post-absorptive data in dog and man. It is of interest, however, that the average value of our observations, representing the extremes of function elicited by the methods used here (and of course unevenly weighted by the number of observations at high and low values) is of the same order of magnitude as is recorded for other mammals (Smith, '51), as shown in figure 1. On the other hand the fraction of water available for facultative excretion in the seal (average 8.2%) is substantially less than that in the recorded data on dogs (12.9%) or man (12.5%) (Smith, '51 and Ludemann, Raisz, and Wirz, personal communication).

McCance ('50) reports several studies indicating that in young animals the urine flow during water diuresis does not attain as high a value as in the adult. This phenomenon appears to be related to the fact that the filtration rate is low in newborn and infantile individuals. We have no information on the maturation of the kidney in the seal, and the fact that facultative water excretion in the animals studied by us is on the average lower than in dog and man may reflect glomerular-tubular imbalance,related to the circumstances that our animals were young. But our feeling is that it more likely reflects a greater conservation of water in the marine mammal. It seems probable that by the time a marine mammal is weaned the kidney has approximately attained the glomerular-tubular balance characteristic of the adult.

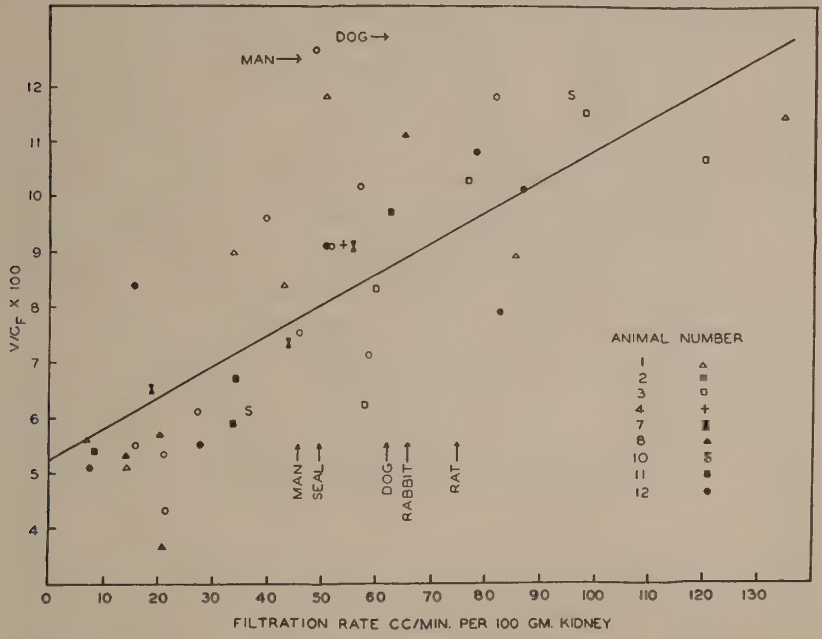

Fig. 1 Linear regression line showing the fraction of filtered water available for facultative excretion in relation to the filtration rate. High filtration rates were obtained after forced feeding, the lowest during the diving reflex. Mean values for filtration rate in man and other mammals are shown along the abscissa; mean values for V/C_F for man and dog are shown in the upper part of the diagram.

The freezing points of plasma and urine were compared at high and low filtration rates in three experiments. The control values of the osmotic U/P ratio were 0.110, 0.132 and 0.095. During the diving deflex, when the filtration rate and urine flow were minimal, these values were 0.116, 0.106 and 0.101. One such experiment is illustrated in figure 2. Thus,

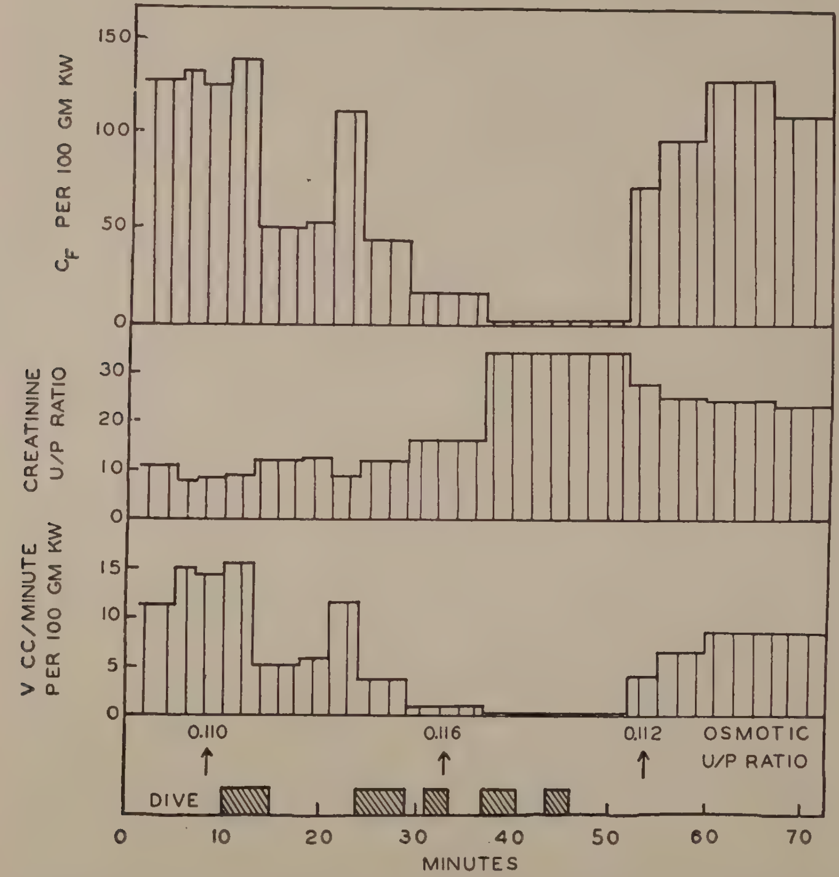

Fig. 2 Changes in urine flow, filtration rate, and creatinine U/P ratio during an intermittent diving experiment, interrupted in order to permit the seal to breathe. A maximal post-prandial water diuresis had been established at zero time. Periods of continuous excitation of the diving reflex induced by closing the nostrils are shown in blocks. The osmotic U/P ratio remained low, despite the reduction in urine flow. The later increased *pari passu* with the filtration rate, beginning shortly after the last "dive.'

reduction in urine flow was not accompanied by any increase in the osmotic U/P ratio, the urine remaining dilute as is typically the case during water diuresis. The fact that the osmotic U/Pratio is not increased at low filtration rates, and at correspondingly low urine flows, indicates that the reduction in urine flow at a low filtration rate is not the result of the secretion of antidiuretic hormone during this reflex, since one would expect the osmotic U/P ratio to have a value of 1.0 or more, if such were the case. Moreover, if the antidiuretic hormone were involved in the reduction of urine flow during diving, one would expect its effect to persist for a short period after the diving reflex is ended. However, we have found that when the reflex is terminated the urine flow may increase *pari passu* with the filtration rate, with no evidence of a persistent increase in the reabsorption of water.

The relationship between facultative water reabsorption and filtration rate, as shown in figure 1, is on the whole similar to that observed in the dog (Ludemann, Raisz, and Wirz, personal communication), except that our data on the seal cover a much wider range of filtration rates. Our data harmonize with the theory that the fraction of the glomerular filtrate (sodium and water) reabsorbed proximally decreases as the filtration rate is increased, with the result that a larger fraction of the glomerular filtrate is delivered to the distal system (presumably as a solution isosmotic with the plasma) where the water is available for excretion as dilute urine after the sodium is reabsorbed. In our present data we find no correlation between the urine flow and chloride excretion. However, our animals were not salt-loaded and chloride excretion was small, indicating nearly complete distal reabsorption of sodium.

Within the range of the minimal creatinine U/P ratios observed here (7.9 to 27.0) the urea/creatinine clearance ratio averaged 0.61 (range 0.22 to 0.87) indicating that in the seal, as in other mammals, there is a deficit in the urea clearance that is attributable to variable passive reabsorption.

SUMMARY

The excretion of water during maximal water diuresis has been examined in the seal, *Phoca vitulina,* at various levels of filtration rate attained by dietary means and by utilizing the diving reflex (which elicits renal ischemia).

At the maximal levels of the filtration rate (approximately 130 cm^3/min. per 100 gm kidney weight) the maximal urine flow during water diuresis represents 12% of the glomerular filtrate. At the lowest levels of filtration rate (approximately 10 cm^3/min. per 100 gm kidney weight) this figure is 5%.

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A COMPLETE ACCOUNT OF THE WATER METABOLISM IN KANGAROO RATS AND AN EXPERIMENTAL VERIFICATION

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SIX FIGURES

A complete account of the water balance of an animal can be given when the water intake and output are known. The water intake can be calculated from the composition of the diet. The total output can be determined when the quantities lost through evaporation, urinary excretion, and defecation are known. Such physiological data for kangaroo rats have been published in previous papers (Schmidt-Nielsen, '48–'50). They are supplemented by data reported in this paper so that the total water balance on a dry grain diet can be accounted for.

Observations on rodents of the family Heteromyidae showed that they usually maintained, or even gained, weight when kept on a diet of dry grain only. However, in certain periods they did not thrive, and slowly lost weight on this diet. Thus, animals kept in captivity in a desert climate (Southern Arizona) tended to lose weight when the humidity ranged from 5 to 10% at 30°C. (about 1.5 to 3 mg H_2O/l air). When the humidity was higher, about 30 to 50% at the same temperature (9 to 15 mg H_2O/l air), the animals gained weight on the same diet. The data were too few to prove that these effects arose from variations in atmospheric humidity, but since the moisture absorbed by the grain as well as the rate of evaporation of water from the lungs of the animals must change with these atmospheric conditions, this explanation of the results was accepted as a working hypothesis.

The ultimate purpose of the investigations of water saving mechanisms in kangaroo rats was to account for the water balance of these animals under any set of conditions. Since the water intake decreases and the water output increases with falling atmospheric humidity, the initial problem was to determine, by calculation, the atmospheric humidity below which kangaroo rats would be unable to maintain a positive water balance on a given dry diet.

Experiments could then be carried out as a check on the calculations and on the data and concepts on which they were based, by keeping animals on a known diet under controlled conditions of temperature and humidity.

CALCULATIONS ON WATER INTAKE AND MINIMUM WATER OUTPUT AT VARIOUS ENVIRONMENTAL HUMIDITIES

The following calculations were based upon data from experiments with Merriams kangaroo rat (Dipodomys merriami, family Heteromyidae). This animal was chosen because the pertinent data on its physiology were more complete than those on the other heteromyids. The various heteromyids show some differences in their ability to conserve water. Pocket mice (Perognathus spp.) seem to have the greater ability, both with respect to concentrate its urine and to reduce its water loss by evaporation. Dipodomys merriami represents an average performance within the group in these respects.

The water intake and water output were calculated for an amount of pearled barley corresponding to 100 calories. (This is equivalent to 25 gm of dry pearled barley.) The quantities of food are expressed in calories so as to avoid confusion due to changes in weight with changing atmospheric humidity. Pearled barley was chosen because the outer indigestible parts of the grain have been removed. When fed other grains the animals selectively leave parts of the grain unconsumed, thereby making it impractical to ascertain the precise composition of the parts consumed.

Water intake

To the animal living on a diet of dry grain, water is available from two sources: (1) Preformed water (water absorbed in the grain), and (2) Oxidation water (water formed by oxidation of the hydrogen in the food, also called metabolic water).

Preformed water. The amount of water absorbed in the grain varies with the relative humidity of the surrounding air. The amount of water absorbed in pearled barley was determined at different relative humidities at 25°C.

Well defined atmospheric conditions were established in desiccators with various saturated salt solutions (spread on the surface of a large amount of the salt to insure a large surface). The following salts were used: $ZnCl_2$ — 10% rel. hum.; $MgCl_2$ — 33% rel. hum.; K_2CO_3 — 43% rel. hum.; NaCl — 76% rel. hum.

TABLE 1

Preformed water

Water absorbed in pearled barley (100 calories) at various humidities

	10% r.h.	33% r.h.	43% r.h.	76% r.h.
Gram of water per	0.90	2.49	2.72	...
100 calories of	0.95	2.57	3.12	4.56
pearled barley	0.93	2.60	2.96	4.50
Average	0.93	2.55	2.93	4.53

The grain was left in the desiccators for not less than three weeks, which was found to be sufficient to reach equilibrium. The water content of the grain was determined in the conventional way by drying to constant weight at 105°C. Three determinations were made at each humidity. The results are given in table 1.

Oxidation water. The amount of water formed in the organism when 100 calories of pearled barley are metabolized can be calculated from the composition of the barley. Our calculations were based upon the composition given in Heinz Nutritional Tables (1946), and the amount of water formed is given in table 2.

Water output

There are three means of water output: (1) Evaporation, (2) Urine formation, and (3) Water loss through feces.

TABLE 2

Oxidation water formed and oxygen required when pearled barley is metabolized

	CARBOHYDRATE	FAT	PROTEIN	TOTAL
Gram of pure foodstuff per 100 calories of pearled barley	22.00	0.28	2.31	
Gram of water formed per gram of foodstuff combusted	0.56	1.07	0.40	
Gram of water formed per 100 calories of pearled barley	12.20	0.30	0.92	13.4
Liter of oxygen used per gram of foodstuff combusted	0.80	2.01	0.95	
Liter of oxygen used per 100 calories of pearled barley	17.60	0.57	2.19	20.4

Evaporation. The problem of evaporation from the Heteromyids was dealt with in a previous paper (Schmidt-Nielsen, '50c). Evaporation from the whole animal (body surface + respiratory tract) was determined and related to the simultaneous oxygen consumption. By determinations on a large number of animals it was found that the evaporation from Dipodomys merriami in dry air is 0.54 mg H_2O/ml O_2 taken up. The individual variations from this average were slight, the standard deviation being 0.04.

On the basis of this value one can calculate the amount of water evaporated from the animal when 100 calories of pearled barley are metabolized and the animal breathes dry air. The amount of oxygen necessary for combustion of the pearled barley is calculated from its composition (table 2). When the 20.4 liter O_2 are taken up by the animal, the simultaneous water loss can be calculated to be 11.0 gm H_2O.

The water loss will be lower than 11.0 gm if the inspired air contains water vapor. Exact determinations of the water loss at various humidities have not been made, but since evaporation from the skin of the kangaroo rat is insignificant, so that the greater part of the total evaporation comes from the lungs, the water loss at higher humidities can be estimated with fair accuracy. This is found by subtracting the water content of the inspired air from that of the expired air. Investigations not yet published have shown that the expired air of rats (kangaroo rats and white rats) is saturated with water vapor at the temperature of the nasal mucosa. This temperature was found to increase with increase in the water vapor content in the inspired air. At a very low humidity, about 1 mg H_2O/liter air, the temperature of the nasal mucosa was found to be between 25 and 26°C. At an absolute humidity of 17.2 mg H_2O/liter air (75% rel. hum. at 25°C.) the temperature of the nasal mucosa averaged 27.7°C. At this temperature saturated air contains 26.7 mg H_2O/liter, and therefore 9.5 mg H_2O are evaporated from the lungs per liter expired air. Assuming that the oxygen content of expired air is 16%, a ventilation of 482 liters is necessary for the metabolism of 100 calories of pearled barley, and the evaporation will therefore be 4.6 gm H_2O per 100 calories of pearled barley when the atmospheric humidity is 17.2 mg H_2O/liter.

Water loss through urine. Since barley contains only a small amount of electrolytes, the amount of urea formed will determine the amount of water required for urine formation when 100 calories of pearled barley are metabolized.

Pearled barley contains 2.31 gm protein per 100 calories (table 2). This gives 0.37 gm nitrogen, or 0.79 gm urea. From this can be calculated the minimum amount of water required for the renal elimination of the urea formed in the organism by the combustion of 100 calories of barley. The maximum concentration of urea that kangaroo rats can excrete is somewhat above 20% (the highest concentration found in any animal was 23%). The specific gravity of a 20% urea solution is 1.05, so that 85 gm H_2O are excreted with 20 gm urea, or 3.4 gm H_2O for 0.79 gm urea. Therefore, the metabolism of 100 calories of pearled barley involves the renal excretion of 3.4 gm H_2O.

TABLE 3

Kangaroo rats

Amount of feces formed on a diet of pearled barley

ANIMAL NO.	DAYS UNDER OBSERVATION	FOOD CONSUMED GRAMS	DRY FECES GRAMS	GRAMS OF DRY FECES PER 100 CALORIES OF PEARLED BARLEY
1	4	14.95	0.557	1.05
2	4	22.23	1.011	1.28
3	4	12.10	0.241	0.56
4	8	32.52	0.652	0.56
5	10	19.55	0.524	0.76
6	14	30.55	0.891	0.82
7	14	36.65	0.872	0.67
8	14	35.85	0.915	0.72
			Weighted average:	0.76

The weight of the food is given as wet weight, i.e., the barley has not been dried before weighing. At the humidity at which the determination was carried out (35% rel. hum.), 100 gm of the pearled barley correspond to 355 calories. The feces were dried to constant weight before weighing.

Water loss through feces. The amount of water lost in the feces when 100 calories of pearled barley is consumed can be found by determination of the quantity of feces eliminated, and its water content.

The amount of feces formed on a diet of pearled barley was determined in individual animals in periods up to 14 days by determining the weight of the food consumed and the dry weight of the feces passed (table 3). The water content of freshly deposited feces was determined in other samples, collected as soon as they appeared. The results of 10 determinations are given in table 4.

From these determinations it can be calculated that when 100 calories of pearled barley are metabolized, 0.76 gm feces (dry weight) are eliminated. This amount of feces would contain 0.63 gm H_2O when fresh.

TABLE 4

Kangaroo rats

Water content of freshly deposited feces

ANIMAL NO.	WET FECES GRAMS	GRAMS WATER PER 100 GRAMS WET FECES	GRAMS WATER PER 100 GRAMS DRY FECES
9	0.0292	53.4	114.7
10	0.0292	48.3	93.3
10	0.0162	43.8	78.0
10	0.0115	49.5	98.2
11	0.0221	43.4	76.8
11	0.0134	41.0	69.6
11	0.0159	44.6	80.7
12	0.0258	43.0	75.5
12	0.0137	45.2	82.6
12	0.0198	39.3	65.0
		Average:	83.4

At what atmospheric humidities can the animals maintain a positive water balance?

Kangaroo rats. The results of the foregoing calculations of the water intake and water output in Dipodomys merriami are represented in a single diagram (fig. 1).

In this diagram the water evaporation is related to the atmospheric humidity, assuming that the relation is represented by a straight line between the two known points. This, of course, is an approximation, but since the significant point, the crossing between the curve for the total intake and the total output is at the left end of the curve, where the error is small, this approximation is sufficiently close for the purpose. The losses through urine and feces are superimposed on this curve, giving the total water loss. Similarly, a curve for preformed water is superimposed on the curve for oxidation water, giving the total water intake.

From this diagram it is seen that the water intake exceeds the minimum water output at all humidities above 2.2 mg H_2O/liter air, or 10% rel. hum. at 25°C. Below this limit the water output exceeds the water intake, so that the animals are in negative water balance.

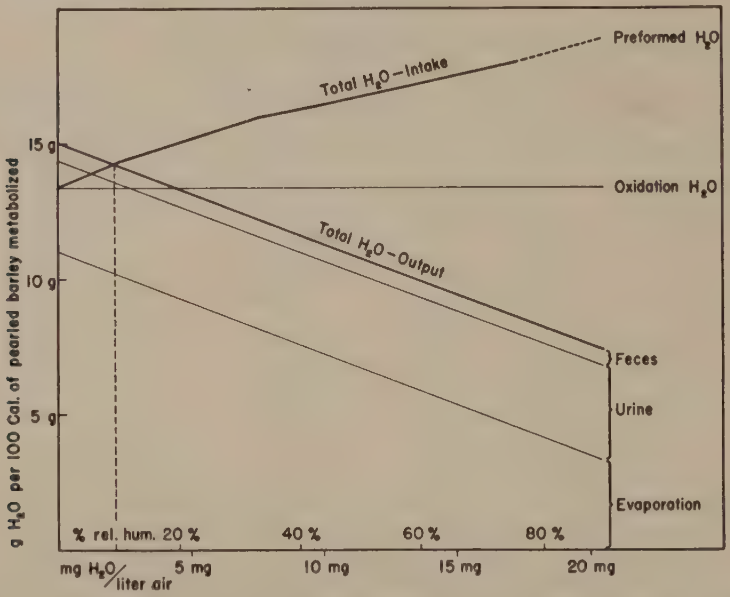

Fig. 1 *Kangaroo rats.* Total water intake and total water output at various atmospheric humidities at 25°C.

Ordinate: Water intake and output in grams per 100 calories of pearled barley metabolized.

Abscissa: Humidity in the environmental air.

White rats. Similar calculations can be made for white rats, so that their water balance can be compared to that of the kangaroo rats.

The evaporation in white rats is calculated from our previous results (Schmidt-Nielsen, '50c), 0.94 mg H_2O/ml O_2, giving 19.2 gm H_2O per 100 calories of pearled barley when dry air is inspired. The slope of the curve that represents the relation to the atmospheric humidity cannot be calculated from our data, since in the white rat the evaporation from the skin is not quite insignificant (Tennent, '46). As a crude approximation the same slope has been used as that determined for the kangaroo rats.

TABLE 5

White rats

Amount of feces formed on a diet of pearled barley. Four animals under observation during a nine day period

DAY OF OBSERVATION	FOOD CONSUMED GRAMS	DRY FECES GRAMS	GRAMS DRY FECES PER 100 CALORIES
1st day	37.74	1.899	1.42
3rd day	76.64	4.839	1.78
5th day	52.68	2.464	1.32
7th day	88.40	4.904	1.56
9th day	96.83	4.860	1.41
		Weighted average:	1.51

TABLE 6

White rats

Water content of freshly deposited feces

ANIMAL NO.	WET FECES GRAMS	GRAMS WATER PER 100 GRAMS WET FECES	GRAMS WATER PER 100 GRAMS DRY FECES
1	0.7672	64.8	183.5
2	0.2509	62.9	168.8
3	0.5138	71.2	246.0
4	1.3315	70.2	237.0
5	1.3448	73.3	288.0
		Average:	224.6

The water loss in the urine is calculated for a maximum urea concentration of 14% (Brokaw, '49), giving a water loss in the urine of 5.1 gm H_2O per 100 calories of pearled barley. The water loss through feces is determined in the manner described previously (tables 5 and 6). From the results it is calculated that 3.4 gm H_2O are lost in feces per 100 calories of pearled barley.

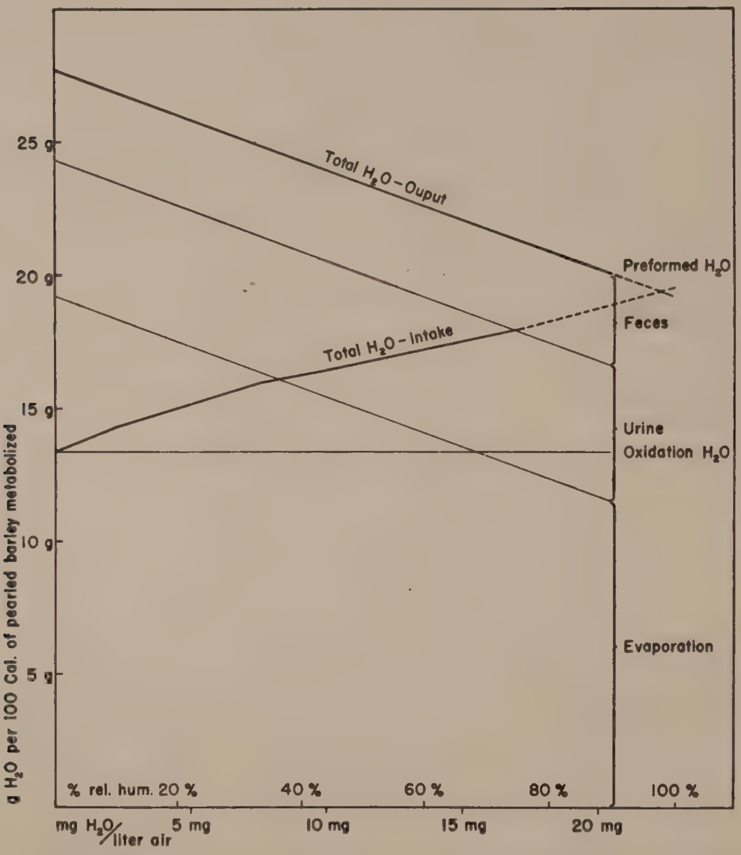

Fig. 2 *White rats.* Total water intake and total water output at various atmospheric humidities at 25°C.

Ordinate: Water intake and output in grams per 100 calories of pearled barley metabolized.

Abscissa: Humidity in the environmental air.

The diagram (fig. 2) shows that the minimum water loss exceeds the water intake at all humidities below 95 to 100%. However, it must be emphasized that the means for arriving at this limit are very uncertain, since the slope of the curve for the evaporation is arbitrarily based on assumptions and not on experimental data. While the error is small in the left part of the curve, the possible error is considerable in the right part in which the high humidities are represented.

ANIMAL EXPERIMENTS AT CONTROLLED HUMIDITY AND TEMPERATURE

Kangaroo rats. In the previous section it was shown how the data on the water output of kangaroo rats can be used to calculate their water economy on a given diet. When fed pearled barley kangaroo rats should be able to maintain a positive water balance at any atmospheric humidity above about 2.2 mg H_2O/liter air. It appeared desirable to check this result by experiments under controlled atmospheric conditions. A reasonable agreement between predictions and actual results would indicate that the approach of investigating the individual water conserving mechanisms as well as the data obtained had been satisfactory.

In order to determine the humidities at which the kangaroo rats are able to maintain a positive water balance, 6 full-grown animals were kept at a temperature of 25°C. and at various controlled humidities. The gain or loss in weight of the animals was used as an indication of positive or negative water balance, respectively. The animals were kept in individual wire cages in a metal chamber ($60 \times 40 \times 25$ cm), placed in a thermoregulated room at 25°C. The humidity was regulated by sending a stream of air (2.5 liter per minute) of the desired relative humidity through the metal chamber. The relative humidity desired was obtained by mixing appropriate proportions of dry air and air saturated with moisture at the same temperature. The streams of air were adjusted to the appropriate proportions before mixing by means of flowrators (Fischer and Porter Co., Hatboro, Pa.). The humidity inside the box was measured by means of microclimate recorders (Krogh, '40), as well as Dunmore electric hygrometers (American Instrument Co., Silver Spring, Md.). The readings gave satisfactory and constant results.

The animals were fed pearled barley which had been kept inside the constant humidity chamber, so that the moisture in the grain was in equilibrium with the moisture in the air. The animals were weighed every other day.

The animals were kept first at 50% relative humidity for 18 days, then after an interruption of 7 days (the compressed air system was out of order), the relative humidity was set at 15% and the animals were kept at this humidity for 12 days. During the following 9 days the relative humidity was maintained at 10%, and thereafter for 9 days at 24%. After an interruption of three months, the experiment was repeated at 5% relative humidity for 7 days.

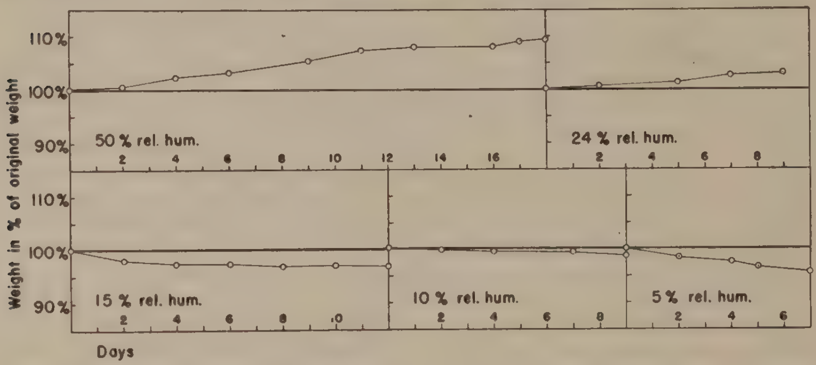

Fig. 3 Weight changes in kangaroo rats on a pearled barley diet at various environmental humidities at 25°C. The curves represent the average weights of 6 animals. The weights are expressed in per cent of the initial weight in each period.

The same 6 animals were used throughout these observations. Thus the animals could be kept in the same place on the same diet throughout the experiments, and the only change introduced in their surroundings was the change in relative humidity.

The curves in figure 3 are plotted from average figures for the 6 animals. The curves for individual animals followed each other closely. The average weights during each period are given in per cent of the average weight of the group at the start of that particular period.

From the curves it is seen that the animals gained in weight at 50% and 24% relative humidity. At 15% the animals lost in weight for a few days after which they maintained their weight. At 10% there was a very slight loss, and at 5% the loss in body weight was more pronounced.

These experiments were repeated later with 6 other kangaroo rats at 45% and less than 5% relative humidity. The results shown in figure 4 are similar to those obtained in the first experiment.

White rats and hamsters. The diagram (fig. 2) indicated that white rats (Rattus norvegicus) might be able to maintain a positive water balance on a diet of dry barley if the humidity were sufficiently high. To investigate this, white rats were kept on a diet of pearled barley without drinking water but in a highly humid atmosphere.

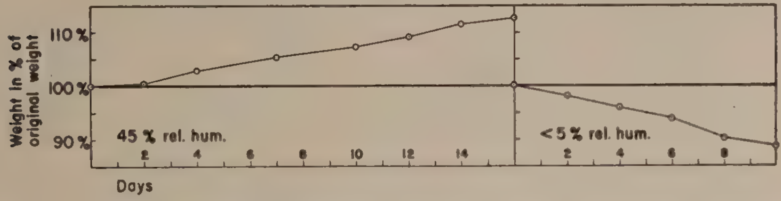

Fig. 4 Weight changes in kangaroo rats at various atmospheric humidities. The experiment is similar to that in figure 3, repeated with 6 other animals.

Hamsters (Cricetus aureus) were included in the experiments because previous experiments (Schmidt-Nielsen, '50c) had shown that they have a comparatively low evaporative water loss (0.59 mg H_2O/mg O_2).

Eight rats were placed in a constant humidity chamber at 90% relative humidity and 25°C. (20.5 mg H_2O/liter air). They were fed pearled barley only. The barley had been kept in the constant humidity chamber for several days in order to reach equilibrium with the atmospheric humidity. A determination of the moisture content of the barley showed that it contained 22 gm H_2O/100 gm dry weight. These rats were kept at this humidity for 11 days and were weighed every three days.

Three rats were kept at 26% relative humidity and 25°C. Their food consisted only of pearled barley.

Two groups of 4 rats each served as controls. Each of these groups was fed pearled barley plus water ad libitum, one being kept at 90% and the other at 26% relative humidity.

The results are given in figure 5. It is seen that, while the two groups given additional water maintained their body weight, the animals on pearled barley without additional water lost weight rapidly. The animals at the higher humidity lost weight at a slower rate than those at the lower humidity.

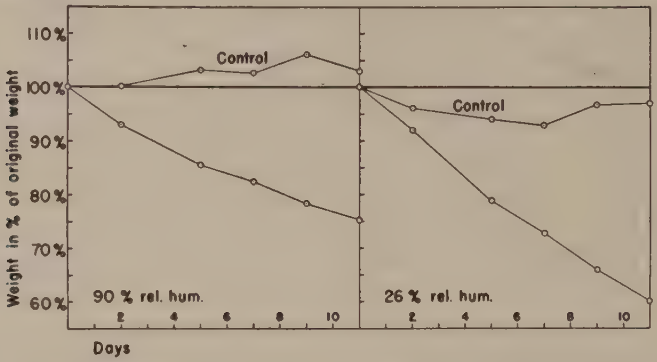

Fig. 5 Weight changes in white rats on a pearled barley diet at 90% and 26% relative humidity at 25°C. The weights are expressed in per cent of the initial weight in each experimental period. The upper curves represent control groups that had access to drinking water.

In the experiment with hamsters 8 animals were kept at 90% relative humidity and 25°C. (20.5 mg H_2O/liter air). Four animals were fed pearled barley without water, the experimental conditions of the other 4 being the same except that they were supplied with water. The results are given in figure 6. It is seen that hamsters also are unable to thrive without water even if the relative humidity is 90%.

DISCUSSION

By calculation it was determined at which environmental humidities kangaroo rats (Dipodomys merriami) can maintain a positive water balance on a diet of dry pearled barley.

The results showed that they should be able to maintain positive water balance at all atmospheric humidities above 2.2 mg per liter air (10% rel. hum. at 25°C.). The validity of the calculations was checked by direct experiments in which the changes in weight of kangaroo rats kept at various humidities were observed. The result of these experiments agreed with the calculations, thereby supporting the validity of the data on the physiological mechanisms and the reasoning on which the calculations was based.

The results also show that, as suspected, the environmental humidity is the determining factor in the water metabolism of the kangaroo rats when they live on dry grain only.

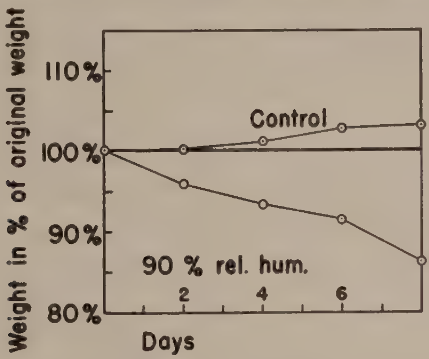

Fig. 6 Weight changes in hamsters on a pearled barley diet at 90% relative humidity at 25°C. The weights are expressed in per cent of the initial weight. The upper curve represents the control group that had access to drinking water.

Our previous determinations of the humidity in the natural habitat of the kangaroo rats (Schmidt-Nielsen, '50a) showed that the humidity in the burrows is considerably above the critical limit, so that even though the animal spends part of his time outside the burrow the overall result will be a positive water balance. The ability of kangaroo rats to survive in the desert without water on a dry seed diet can therefore be fully explained by their physiological mechanisms for water conservation.

The calculations on the water intake and water output of white rats indicated that they might be able to maintain positive water balance on the dry grain diet if the atmospheric humidity were around 20 to 22 mg H_2O/liter air, (close to 100% rel. hum. at 25°C.). Direct observations demonstrated that white rats and hamsters are unable to thrive on a dry grain diet without drinking water even at very high atmospheric humidities.

This marked difference in the ability to conserve water, between the kangaroo rats on one hand, and the white rats and hamsters on the other, shows how well adapted the kangaroo rats are to their desert habitat.

SUMMARY

A complete account of the water intake and water output of kangaroo rats while maintained on a dry grain diet is presented.

The calculations are based upon data on evaporative water loss and urine concentration in kangaroo rats presented in previous publications and on additional data on water loss through feces and moisture determinations in pearled barley fed to the animals.

The account shows that kangaroo rats are in positive water balance when given a diet of dry pearled barley without drinking water at all atmospheric humidities above 2.2 mg H_2O/liter air (10% rel. hum. 25°C.).

The result of the calculations was checked by direct experiments. Kangaroo rats were kept at various atmospheric humidities and their weight changes were registered. The animals could not maintain body weight below about 2.5 mg H_2O/liter air, but above this limit they maintained or gained body weight.

An approximate account of the water intake and water output in white rats on the pearled barley diet is presented. The water output in white rats is considerably higher than in kangaroo rats and according to the calculations, they are in negative water balance at all atmospheric humidities below

around 21 mg H_2O/liter air. White rats and hamsters kept at atmospheric humidities around 21 mg H_2O/liter of air were not able to maintain body weight but lost weight rapidly.

ACKNOWLEDGMENT

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A METHOD FOR THE IN VITRO COLLECTION OF PURE GASTRIC JUICE FROM ISOLATED GASTRIC MUCOSA

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FOUR FIGURES

The study of isolated cells or tissues has certain advantages over studies using intact animals or whole organs. Conditions can be more completely controlled and indirect influences can be eliminated. However, the in vitro technique has the problem of maintaining the cells in a normal, physiological environment in which normal function can be maintained.

The technique to be described is a means by which isolated secretory gastric mucosa can be studied. Delrue, in 1930, and Gray et al. ('40) have shown that it is possible to obtain acid secretion from the isolated gastric mucosa of the frog. However, their methods did not permit collection or measurement of the unmodified secretion but only the change in acid concentration in a salt solution to which the secretory cells were exposed. The present method (Edwards and Edwards, '48, '49) was designed to allow repeated collections of unmodified secretion. Davies ('48) used sacks of mucosa that filled with secretion which could then be studied. Only one sample can be obtained by this method. Gas exchange studies can be done simultaneously with the secretion. The sack method has also been used by Davenport ('48) using mouse mucosa.

METHODS

The essential design of the apparatus[1] consists of two chambers separated by a membrane of living secretory tissue. The top chamber contains a nutrient medium; the unmodified secretion drains into the lower chamber from which it is collected. The design is such as to satisfy all conditions for the survival of living tissue. It also allows for rapid mounting of the tissue which is clamped between the two chambers tightly and evenly enough to prevent leakage but not so as to cut the surrounding tissue as this also causes the development of a leak.

Apparatus. In shape the glass nutrient chamber closely resembles a Warburg flask with a hole in the bottom. This chamber is provided with an inlet and an outlet tube for gas mixtures. The inlet tube is so shaped that most of the fluid can be removed by suction if the flask is inverted or tipped to the appropriate angle. The bottom of this chamber has a ground glass flange which allows for the mounting of the tissue between the top and bottom chambers. See figure 1.

The bottom or collecting chamber is essentially a glass funnel provided with an inlet and an outlet tube. Both these tubes project into the funnel and then slope downward so that there is no tendency for the juice to collect in these side tubes. The top of the funnel-shaped chamber has a ground glass flange for clamping the two chambers together.

To the stem of the funnel a small, replaceable cup is attached by means of a rubber stopper. To facilitate drainage by preventing air traps, a gas outlet from the cup is also provided. A syringe needle through the rubber stopper and connected to the funnel outlet has proved adequate. See figure 1.

The two chambers are clamped together with the tissue membrane between the ground surfaces. The clamp is made of two aluminum collars, each made to slip over the corresponding chamber and rest against its flange. Three, equally spaced, aluminum bolts are used to clamp the two flanges together. In order to provide for changes in expansion due to temperature variation and to assure even tension on all bolts, a small coil spring, no. 27 (1½″ × 3/16″) is slipped over each bolt between the wing nut and the collar and tightened to ¾″. Thus it is the force of the compression spring which holds the flanges together. The flanges therefore exert a constant pressure on the tissue membrane which is the same

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[1] Some preliminary designs of the apparatus were made in 1947 while one of the authors (L.E.) was working with the Fel's research group in the Philadelphia laboratories.

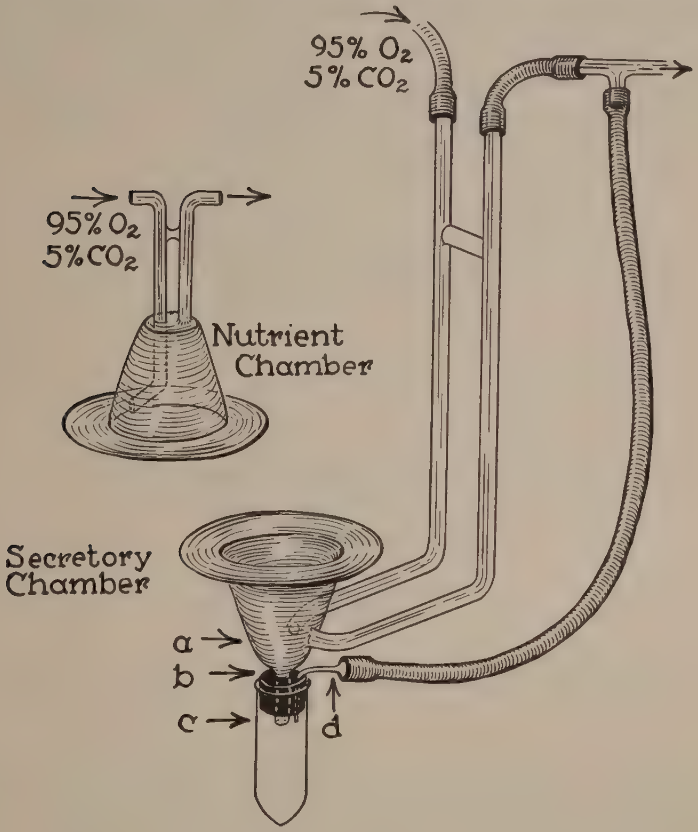

Fig. 1 Nutrient and secreting chambers; a. glass collecting chamber, b. rubber stopper, c. collecting tube, d. bent syringe needle.

for all experiments. Thin rubber rings are used to protect the flanges from the metal collars. See figure 2.

The holder for the glass chambers was made to fit the ordinary shaking equipment used for the Warburg respirometers. All the metal parts were made from aluminum. This holder is shown in figure 3. The stand used for mounting the tissue also resembles the stand used for mounting the manometers for Warburg respirometers.

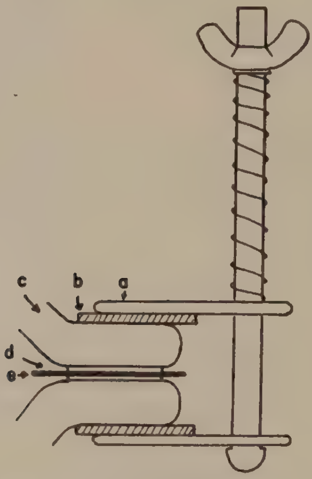

Fig. 2 Clamp; a. aluminum collar, b. rubber washer, c. glass flange, d. celluloid disk, e. tissue membrane.

A gas mixture was supplied to each side of the tissue. The gas pressure above the membrane was 11 cm of water while that on the under side was 1 cm. Thus there was a constant pressure gradient of 10.4 cm of water between the top and bottom surfaces of the membrane of which .4 cm was due to 5 cm^3 of the medium. Pressures were governed by water manometers set up as shown in figure 4.

Solutions. The physiological salt solution worked out for the frog by Gray ('40) was used for many of the first bullfrog experiments. Heparinized bullfrog blood was used in some of the later work. In the mammalian experiments, serum was used in the majority of cases. Blood was drawn from the animal under CO_2 anesthesia prior to the removal of the stomach. For Warburg determinations of tissue respiration at the completion of the secretory experiments Ringer's-phosphate was used. For amphibian experiments this was Gray's solution with phosphate substituted for the bicarbonate in an amount such that the osmotic pressure was unchanged.

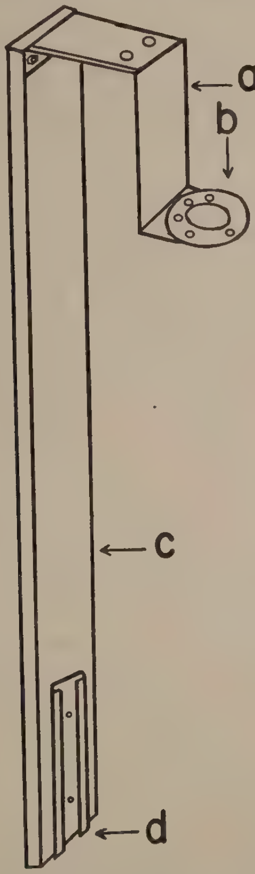

Fig. 3 Mounting; a. aluminum support, b. lower collar of clamp, c. wooden support, d. holder.

Gas mixtures. All solutions were equilibrated with 95% O_2 + 5% CO_2 during the secretory periods. One hundred per cent O_2 was used to equilibrate the solution used in the Warburg flask as no bicarbonate was present in the solutions.

Preparation of the tissue. In nearly all the experiments 40% CO_2 + 60% O_2 was used to anesthetize the animals. In a few bullfrog experiments in which no blood was drawn, the bullfrogs were pithed rather than anesthetized.

The stomach was removed from the animal and quickly washed free of all stomach contents in ice-cold Ringer's-phosphate, appropriate to the animal involved. In mammalian experiments it was found necessary to dissect the tissue over ice.

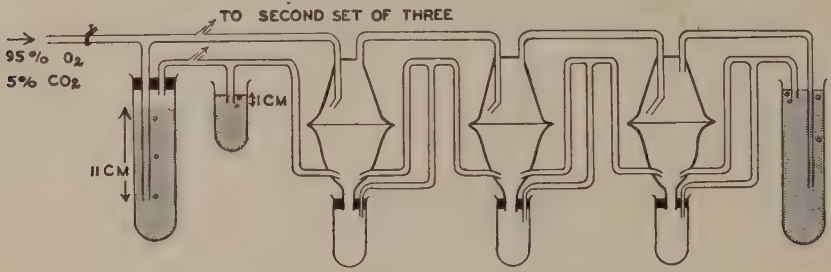

Fig. 4 Diagram of gas supply attachments.

The muscle layers were first cut away from the mucosal layers with forceps and fine scissors, separating in the submucosa as close as possible to the mucosa. In the dog, the muscularis muscosae can also be separated from the epithelium. This is done by laying the partly dissected tissue, lumen side down, on a clean, cold, glass plate and gently pulling away the connective and muscle tissue with forceps while holding the epithelium at the edge by pressing it against the plate. Some experience is necessary to judge the tension required and to remove the unwanted tissue without tearing the epithelium. In some individual animals (usually the older ones) it is found to be impossible, but in most cases the cleavage line is distinct and the separation can be made. When the dissection is complete, a thin sheet of tissue remains with a smooth surface, very similar to the free, lumen surface.

The tissue, prepared as described, was blotted on filter paper to remove all secretory material from the lumen side.

Mounting. The glass chambers were assembled on the mounting and the rubber tubes for connections were added. Only two of the three bolts were put in place, and these were left as loose as possible. Both flanges were lightly greased or waxed.

The dissected membrane was placed between two lightly greased or waxed celluloid rings of the same size as the flanges. This greatly facilitated handling and mounting the tissue. The two rings with the sheet of tissue, cut to a size slightly larger than the rings, were picked up with forceps and the whole was slipped between the two glass chambers which were held slightly apart. The third bolt was then inserted, thus holding the tissue loosely. At this time the membrane may be stretched taut by pulling the protruding edges with the forceps. The nuts were then screwed down to a set distance (¾″) from the collar.

Nutrient medium (usually 5 cm^3) was added to the top chamber through the gas inlet tube, using a syringe. Each mount was then placed in the water bath, mounted on the shaker. When all were in place the gas tubes were connected between them with small pieces of glass tubing. The gas line was also connected and a screw clamp so adjusted that a slow stream of bubbles emerged at each of the manometer outlets.

Collection of juice. The juice was collected in the bottom cup for a period of 2–3 hours for mammalian tissue. In bullfrogs the secretion was followed until free acid production had ceased. In some cases this extended over a period of 20 hours. In these cases the collection cups were replaced every 2 to 3 hours, depending upon the secretion rate.

Final treatment of the tissue. At the end of the secretory period, samples were cut from the mucosa and the areas and dry weights were measured. From these data the thickness could be calculated in the standard manner.

The oxygen uptake of other samples of tissue was measured in most of the experiments in order to determine the viability of the tissue. The standard Warburg technique was used.

Analyses. Preliminary tests for the presence of acid in the secretion were made with universal indicator paper. For bullfrog secretions, pH was measured with the Beckman pH meter and free acid and total acid were determined by electrometric titration to pH 3.5 and 8.5 respectively, using 0.01 N NaOH.

Pepsin was initially measured by the Mett method but later the hemoglobin digestion method of Anson and Mirsky ('33) as modified by Bucher, Grossman and Ivy ('41) was used. The latter gives more consistent and reliable values.

RESULTS

The technique was tested on a number of species. Of the animals tested, the bullfrog and the dog were by far the most satisfactory. Both gave membranes of adequate size and tensile strength. The dog stomach is especially good because of the more complete isolation of the epithelium, mentioned above, which was confirmed by histological sections. In other species the muscularis mucosae can sometimes be separated, but the epithelium tears in the process.

Other common laboratory animals proved less satisfactory. The rabbit can be used but dissection is difficult and leaks often develop. Under the conditions used no acid is secreted and only small amounts of pepsin are secreted without some type of stimulation. A few trials of dissection of the cat stomach showed that the mucosal membrane of the cat is structurally similar to that of the rabbit. It is possible, therefore, that the cat could be used, although it is not the animal of choice. One young goat was tried and gave adequate membranes.

As may be seen from the table, the volume rate of secretion (cm^3/hr./unit area) is variable, both among species and among individuals of the same species. However, in general, the order of magnitude (0.1–0.7 cm^3/hr./10 cm^2) [2] remains the same. There are no significant species differences. It is higher when an artificial medium is used than when blood or serum

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[2] The center hole in the celluloid rings exposes an area of 10 cm^2 of the mucosa.

TABLE 1

"Range" gives the extreme values obtained; "Average" gives the arithmetic mean of all the values obtained, plus or minus the standard error of the mean. The "Total acid" column gives the total mM of acid (HCl) secreted throughout the experiment, which continued until free acid secretion ceased. These values are calculated from titration figures.

Pepsin concentration is given in Mett units with the exception of those figures marked with an asterisk. The latter are in "Pepsin Units" of Anson and Mirsky

SPECIES	NUMBER		MEMBRANE	NUTRIENT SOLUTION	SECRETION								
					Appearance	Rate cm³/hr./10 cm²		Acid				PEPSIN CONCENTRATION METT UNITS (*PEPSIN UNITS)	
								Max. conc. mM/L.		Total mM.			
	Animals	Pieces				Range	Average	Range	Average	Range	Average	Range	Average
Bullfrog	7	7	Fairly good	Ringer's	Clear, viscous	.9–2.4	1.3 ± .019	.003–.073	.034 ± .008	.003–.046	.027 ± .005	0–4.9*	2 determinations each
	4	4		Blood		.08–.18	.14 ± .03	.051–.107	.077 ± .010	.031–.108	.085 ± .017	10–12*	
Dog	7	29	Very good, Muscularis mucosae removed	Serum	Clear, viscous	.06–.87	.34 ± .04	None				.6–13.6*	2.5 ± .5*
	7	28				.11–1.55	.68 ± .09					1–64	23 ± 3.4
Rabbit	6	6	Not very good	Serum	Dark, viscous	.03–2.00	.78 ± .25	None				0–16	4 ± 4
	10	23		Serum + drugs		.01–.81	.17 ± .05					0–400	78 ± 17
Rat	2	2	Too small, peels well	Saline	Dark, viscous	.33–.72	.53 ± .14	Sometimes slightly acid to indicator paper				0–52	26 ± 18
	8	8		Serum		.13–.47	.29 ± .04	None				27–1023	578 ± 146
Turtle	1	1	Fairly good	Ringer's	Thin, clear	.14–1.56	.68 ± .36	None				0–16	5.3 ± 4.4
	2	2		Serum									
Goat	1	3	Good	Serum	Clear, viscous	.07–.18	.11 ± .03	None				288–389	326 ± 26

is used as the nutrient medium. It is of such a magnitude that secretion may be collected in volumes adequate for subsequent analyses.

Of the animals tested, only the bullfrog gave acid secretion. The initial pH of the secretion was usually between 1 and 2, though values below pH 1 have been obtained. Acidity was higher and was maintained for a longer period of time when blood was used as a nutrient medium in place of Ringer's. The results of these and similar, later experiments will be reported in detail in a subsequent paper.

Some peptic activity was found with most species though most values for unstimulated mucosae were relatively low. A few preliminary tests with stimulating agents indicate that much higher peptic activity can be obtained with the parasympathetic drugs.

Q_{O_2} studies done on samples of the mucosa after the completion of the secretory experiment (2 hours) show that dog mucosa is still viable and respiring normally at the end of this time. Q_{O_2} values averaged $10.8 \pm .34$[3] and were linear as measured over a period of 45 minutes. The lower values were usually associated with a falling oxygen consumption and with tissue thickness greater than 0.45 mm. Such tissues usually gave low volumes of secretion and low peptic activity. Oxygen consumption of tissue pieces after the secretory period paralleled those of tissue pieces shaken in the Warburg flask during the same period.

DISCUSSION

The apparatus described in this paper was designed to fulfil the requirements used in the standard Warburg technique in respect to shape of flask, rate of shaking, manner of shaking, aeration of the flask and thickness of the tissue. Therefore, the aeration of this tissue on the submucosa side should be comparable with that in the Warburg flask. On the bottom side of the membrane, only the secreted film exists for the exygen to diffuse through to reach the tissue cells.

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[3] The standard error of the mean.

The oxygen diffusion constant for the stomach has not been worked out. Nevertheless, one can calculate the limiting thickness for oxygenation of the mucosa to a very close approximation. Krogh ('30) worked out the diffusion constant for several tissues. The diffusion constant for the peritoneum, a tissue closely related to the mucosa, is given as 1.36×10^{-5} at 38°C. for the dog. The diffusion constant for oxygen through a membrane is defined as the number of cubic centimeters (0°C., 760 mm) penetrating through 0.001 mm (1 μ) thickness and 1 cm^2 surface per minute when the pressure difference is 1 atmosphere. Most workers have used the diffusion constant for muscle (1.4×10^{-5}) in calculating the limiting thickness for other tissues. These values are so nearly identical that only very slight error would be made in assuming the diffusion constant for muscle.

If one uses the limiting thickness formula worked out by Warburg ('23), the limiting (d^1) can easily be calculated. The formula is given by the following equation: $d^1 = \sqrt{8\, C_o \times \frac{D}{A}}$ where C_o is the oxygen concentration outside the slice, measured in atmospheres (.95), A is the rate of respiration $\frac{(\text{ml } O_2 \text{ uptake})}{(\text{ml tissue} \times \text{min.})}$, D is the diffusion constant for oxygen in ml. (N.T.P.)/cm^2/μ/min./atm. Q_{O_2} is corrected to A by multiplying by 1/300, assuming that the ratio of dry weight to wet weight is 1/5. The experimental figure was found to be 1/5.04 in four experiments. The oxygen quotient (Q_{O_2}) is defined as the number of μl. of O_2 taken up per mg dry weight of tissue per hour.

When the average Q_{O_2} for the dog mucosa of 10.8 was used, the limiting thickness was calculated as 5.36×10^{-2} cm or .5 mm. For the bullfrog, for an average Q_{O_2} of 2.01, the limiting thickness was calculated as 1.2 mm for a D of 1.23×10^{-5} calculated for a temperature of 25°C. In general, the thicker membranes from the same animal have lower Q_{O_2}'s than the thinner ones. From general observation it appears that the thicker membranes have more connective tissue than do the thinner ones. This lower ratio of secretory tissue to total tissue would explain in part the lower oxygen quotients found.

As described in the methods, two different separations of the mucosa were possible. In the first, the mucosa and part of the submucosa made up the membrane. The membrane consisted of two layers and part of a third; the mucosal epithelium, the muscularis mucosae and some connective tissue of the submucosa. The second membrane consisted of only the epithelial mucosa containing the secretory glands. As the epithelial mucosa of most animals does not have sufficient tensile strength to form a sheet after separation from the other layers, only the first method of separation was used in most species. Nonetheless, the incomplete separation gives a sheet of tissue which is predominantly secretory and can be used for some of this work. The second method was used most successfully with dogs. Since this leaves essentially a single secretory membrane, this type of membrane is the desired one.

Since lowered temperature lowers the metabolism and therefore lowers the oxygen consumption of the tissue, the separation of the mucosal membrane was done over ice for most species. The effect of temperature on the rate of secretion and on the total amount of pepsin will be reported in a later publication.

One indication of a normal, functioning tissue is the constancy of its oxygen consumption. As a tissue dies its metabolism falls and ultimately reaches zero on complete death of the tissue. The greater the damage to the tissue the sooner the tissue dies and the more rapidly the oxygen consumption falls. Therefore, we think of the falling oxygen metabolism as an indication of dead or dying tissue. It is interesting to notice that the dog mucosa maintains a very constant level of metabolism for several hours. This is an indication that the tissue is normal for that period.

When tissue does not receive sufficient oxygen it also dies. This can be demonstrated in pigeon breast muscle studies. This tissue has a high oxygen uptake and will die during the preparation of the tissue unless the tissue is chilled over ice.

The fact that the tissue maintains a constant oxygen uptake indicates also an adequate oxygen supply.

In *in vitro* studies the problem of anesthesia becomes a major one. In smaller animals such as the frog, pithing can be used. Thus procedure works adequately when only tissue is to be removed. If blood is also taken, some type of anesthesia is preferred.

As for the larger animals such as the dog, the animal may be killed by a blow on the head. The time involved in removing the tissue then becomes critical. If the removal of blood is required, more valuable time is lost and there may be difficulty in obtaining enough blood.

The anesthesia chosen for these experiments was a mixture of 40% CO_2 and 60% O_2. This anesthetic acts very quickly and if allowed to do so the animals recover very quickly. From a physiological point of view no foreign material has been added to the animal. The mechanism for CO_2 removal is very efficient and in these experiments should act in the same fashion as the removal of CO_2 from the normal tissue. Since the dissected mucosa is very thin it will quickly come into equilibrium with its environment and no lasting effects of the anesthetic should remain with the tissue. For this reason CO_2 is an excellent anesthetic for tissue study.

In most tissue work, no anesthetic is used as the animals are killed by a blow on the head. The CO_2 and O_2 mixture allows the tissue to continue receiving blood and oxygen during the removal and thus alleviates some of the need for haste. This anesthetic allows for the withdrawal of blood just prior to the final dissection of the mucosa.

As mentioned earlier, Gray's Ringer's was used in the early bullfrog studies. Glucose was also added to the solution. Gray had worked out the optimal conditions for the frog mucosa. It was found, however, that the amout of fluid secretion was higher in Gray's Ringer's than with heparinized blood or serum for the bullfrog. This added secretion was due to a change in permeability of the membrane and not to improved acid secretion. More details will be given in a subsequent paper.

In this investigation the study has been confined to the unstimulated preparations. The lack of acid secretion in many of the different species of animal should not be disturbing because the dog, cat, rabbit and probably several of the other species used have no spontaneous acid secretion. The frog and the bullfrog have a spontaneous acid secretion. Therefore, no stimuli for acid secretion are necessary in these species. This is essentially, therefore, a study of basal secretion. The basal secretion studies are less complicated than the stimulated ones, as the variations of the action of the stimuli are avoided.

CONCLUSIONS

1. This technique offers a valuable tool for studying pure gastric secretion in vitro.
2. The isolated mucosa offers a membrane that can be studied under rigidly controlled conditions.
3. The amount of basal secretion is adequate for many analytical studies.
4. Several samples are possible from the same preparation.
5. The most useful membranes are those obtained from the dog, bullfrog and goat.
6. The bullfrog is the best animal of those studied for studying spontaneous in vitro acid secretion.
7. The limiting thickness has been calculated for the dog as .5 mm and for the bullfrog as 1.2 mm under the conditions described.

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ASPHYXIAL POTASSIUM LOSS OF MAMMALIAN NERVE[1]

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Fenn ('40) reported that frog nerve immersed for 5 hours in Ringer's solution does not lose more potassium to the surrounding fluid in the absence of O_2, than when oxygenated. However in a more recent investigation, Fenn and Gerschman ('50) found less potassium in frog nerve asphyxiated for 1 to 42 hours at 4°C., than in nerves which were kept oxygenated for the same periods of time. The potassium losses were quite variable and were not obviously related to the duration of asphyxiation. When the nerve after asphyxiation was kept for some time in oxygenated Ringer's solution, the lost potassium tended to return to the nerve. Due to their low metabolism, cold blooded animals may not render the most favorable material for studies of this kind. In warm blooded material (rabbit) van Harreveld ('50) observed a considerable loss of potassium from the nerve during a 6 hours asphyxiation. Such a period of asphyxiation is relatively long for a mammalian tissue, and therefore the loss of potassium might either be caused by structural damage to the nerve or by the arrest of a potassium accumulating process which requires energy. An investigation into the effects of shorter periods of asphyxiation on the potassium content of rabbit nerve seemed therefore of interest.

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METHOD

All experiments were performed on rabbit's sciatic nerves which weighed from 150 to 200 mg depending on the size of the animal. The Ringer's solution used contained .9% NaCl, .038% KCl, .02% $CaCl_2$, .02% $NaHCO_3$. To the solution was added 0.2% glucose when needed.

After removing adhering fluid with filterpaper the nerve was weighed in a platinum crucible, dried at about 100°C. to constant weight, and ashed at 500°C. The ash was dissolved in 1.2 cm^3 5% acetic acid, and the potassium determination was performed in 1 cm^3 of this solution, using a modification of the colorimetric procedure of Shohl and Bennett ('28). In the majority of the experiments, the K concentration in the test solution was between 15 and 25 mg %. The probable error computed from 8 determinations of a 20 mg % solution was 2.5%. The potassium content of the nerve was computed on the basis of wet and of dry weight and rounded to the nearest mg %. The percentage changes and probable errors were rounded to the nearest 0.5%.

RESULTS

Potassium losses in oxygenated nerve. In 8 experiments (table 1, A) one sciatic nerve was dried immediately after removal from the animal, the heterolateral nerve was kept for 2 hours at 38°C. in Ringer's solution with substrate (0.2% glucose), oxygenated with 5% carbondioxide in oxygen. The experimental nerves were found to have sustained a loss of 11.5(± 2.5)% of their potassium computed on the basis of the wet weight of the nerves, and 12.5(± 3)% on the basis of the dry weight. This loss is significant (at the 1% level). When the same experiment was performed, but the experimental nerves were kept in the oxygenated Ringer's solution for 6 instead of 2 hours (table 1, B), the loss of potassium was 7(± 2.5)% computed from the wet figures and 8.5(± 2)%

using the dry weight figures.[2] A comparison of these two groups of experiments seems to indicate that the potassium is lost during the first hours the nerve is bathed in oxygenated Ringer's solution. During the ensuing hours no loss is sustained; the nerve may even regain some of the lost K. This is confirmed by an experiment again on 8 pairs of nerves (table 1, C) in which the two nerves of each rabbit were kept for 4 hours at 38°C. in Ringer's solution with substrate, oxygenated with 5% CO_2 in oxygen. Then one of the nerves was dried. The other was left in the Ringer's solution for an additional 4 hours, and then dried. The latter nerves showed a difference of $-2(+2.5)\%$ on the basis of the wet weight, and no change of the potassium contents $(\pm 3\%)$ based on the dry weight figures. This experiment indicates also that after the first hours the nerve does not change significantly in K content during a subsequent stay in oxygenated Ringer's solution.

Potassium losses in asphyxiated nerve. In 8 experiments (table 1, D) one nerve was dried immediately after removal from the animal, the heterolateral one was kept for 2 hours in Ringer's solution at 38°C. without substrate, bubbled through with 5% CO_2 in nitrogen. The potassium loss was $22.5(\pm 3)\%$ based on the wet weight and $19.5(\pm 3)\%$ on the dry weight figures. The potassium loss during a 2 hours asphyxiation was greater than when the nerve remained for 2 hours in oxygenated Ringer's solution (table 1, A). The difference of the dry weight figures of these two experiments is not statistically significant; the difference between the wet weight figures is significant at the 5% level.

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[2] It was found previously (van Harreveld, '50) that rabbit nerve kept under similar conditions for 6 hours did not change significantly in K content. The statistical treatment of the figures of the present experimental series and those of the series reported previously shows them not to be significantly different. Furthermore the K content of the Ringer's solution in the present experiment was slightly lower (20 mg %) than in the experiment reported before (21 mg %). This lower K concentration was chosen in the present investigation since it approaches closer the mean potassium concentration of rabbit's blood.

TABLE 1

EXP. SERIES	NUMBER OF EXP.	CONTROL (MG % K)		TREATMENT	TREATED (MG % K)		MEAN DIFFERENCE (%)	
		Wet	Dry		Wet	Dry	Wet	Dry
A	8	202	585	Control nerve no treatment. Experimental nerve 2 hours in Ringer's with 0.2% glucose and 5% CO_2 in O_2.	179	510	— 11.5 (± 2.5)	— 12.5 (± 3)
B	8	194	603	Control nerve no treatment. Experimental nerve 6 hours in Ringer's with 0.2% glucose and 5% CO_2 in O_2.	180	553	— 7 (± 2.5)	— 8.5 (± 2)
C	8	180	513	Control and experimental nerve 4 hours in Ringer's with 0.2% glucose and 5% CO_2 in O_2. Experimental nerve 4 additional hours in the same medium.	176	514	— 2 (± 2.5)	0 (± 3)
D	8	185	541	Control nerve no treatment. Experimental nerve 2 hours in Ringer's with 5% CO_2 in N_2.	143	433	— 22.5 (± 3)	— 19.5 (± 3)
E	8	196	560	Control nerve no treatment. Experimental nerve 2 hours in Ringer's with 0.2% glucose and 5% CO_2 in O_2, followed by 2 hours in Ringer's with 5% CO_2 in N_2.	145	415	— 26 (± 2)	— 26 (± 2)
F	8	188	538	Control and experimental nerve 4 hours in Ringer's with 0.2% glucose and 5% CO_2 in O_2. Experimental nerve an additional 2 hours in Ringer's with 5% CO_2 in N_2.	154	458	— 18 (± 3.5)	— 15 (± 3.5)
G	8	180	512	Control and experimental nerve 4 hours in Ringer's with 0.2% glucose and 5% CO_2 in O_2. Experimental nerve an additional 4 hours in Ringer's with 5% CO_2 in N_2.	127	385	— 29 (± 3.5)	— 24.5 (± 3.5)

In a series of experiments (table 1, E) the nerve on one side of the animal was dried immediately after removal. The heterolateral nerve was kept for 2 hours in Ringer's solution with substrate oxygenated with 5% CO_2 in O_2. Then the nerve was kept for 2 hours in Ringer's solution without substrate, bubbled through with 5% CO_2 in N_2. The experimental nerve loses 26(± 2)% computed from the wet weight figures, and also 26(± 2)% based on the dry weight figures. It was shown above that the K loss of nerve in oxygenated Ringer's solution occurs during the first hours, and that no significant additional losses are sustained during a subsequent stay in the same medium. The increased loss of potassium in this experiment as compared with the loss during a 2 hours stay of the nerve in oxygenated Ringer's solution (table 1, A) therefore must be due to the 2 hours asphyxiation. This increased loss is 14.5% computed on the basis of the wet weight, and 13.5% on the dry weight figures. This extra asphyxial loss is significant at the 1% level for the wet weight figures, and on the 5% level for the dry weight figures.

In another series of experiments (table 1, F) the left and right nerves were left in oxygenated Ringer's solution for 4 hours. Then one nerve was placed in Ringer's solution without substrate at 38°C., bubbled through with 5% CO_2 in N_2, this was continued for 2 hours. The other nerve, serving as control, was dried. The experimental nerve showed a loss of 18(± 3.5)% of its potassium, based on the wet weight figures, and of 15(± 3.5)% computed from the dry weight figures. It was found that nerves after a stay of 4 hours in oxygenated Ringer's solution do not lose potassium during an additional period of 4 hours in the same medium (table 1, C). The marked K loss of the experimental nerve, therefore, must be ascribed to the asphyxiation. A comparison of the figures of these two experimental series (table 1, C and F) shows this loss due to asphyxiation to be significant on the 1% level for the wet weight figures; for the dry weight figures the loss although clearly significant on the 5% level just fails to reach significance on the 1% level. The same experiment was repeated, asphyxiating the experimental nerve for 4 hours instead of two (table 1, G). The losses were 29(± 3.5)% based on the wet weight and 24(± 3.5)% based on the dry weight figures. The losses of potassium are greater in this experiment, commensurate with the longer period of asphyxiation.

DISCUSSION

A significant loss of potassium was detected when the isolated sciatic nerve was kept for 2 hours in oxygenated Ringer's solution. No losses were sustained during subsequent hours in the same medium. Such an initial loss of K was described by Arnett and Wilde ('40) and by Fenn and Gerschman ('50) for isolated frog nerve kept in oxygenated Ringer's solution. This loss may be caused by diffusion from the cut ends of the nerve. Fenn and Gerschman found, however, that tying off the cut ends increases rather than decreases the K loss. Another possibility is that the initial K loss is due to the activity of the injury potential, which may cause a migration of sodium ions from the surrounding medium into the cut ends, allowing a release of potassium ions through the mantle of the nerve fiber.

In the experimental series D, in which the nerve was asphyxiated for 2 hours immediately after removal from the animal, the potassium loss is probably caused in two ways. As long as asphyxiation does not exert its effect, the nerve may lose K by the mechanism which causes a freshly isolated nerve to lose potassium in oxygenated Ringer's solution. After asphyxiation takes effect a loss due to the O_2 lack may occur. Such an experiment therefore does not allow the estimation of the loss due to asphyxiation. This difficulty has been overcome in the experimental series E, F and G in which the experimental nerve was first kept for 2 to 4 hours in oxygenated Ringer's solution and then was asphyxiated. The increased loss of potassium as compared with the appropriate control series (A for series E, and C for series F and G) can be considered as the true loss of K due to asphyxiation. The K losses of asphyxiated nerve computed on the basis of the wet weight figures tend to be slightly higher than those computed from the dry weight figures. This indicates that the experimental nerve takes up some water from the medium. The dry weight figures therefore should reflect the K losses due to asphyxiation more truthfully than the wet weight figures.

Significant potassium losses were found even for the relatively short asphyxial period of 2 hours. This observation is of interest, since Wright ('47) found that the decline of the membrane as well as of the action potential during a 2 hours asphyxiation is fully reversible. Wright found furthermore that the membrane potential started to recover 1 to 3 minutes after the beginning of renewed oxygenation, which makes it highly unlikely that serious structural damage is incurred by the nerve. The loss of potassium during a 2 hours asphyxiation is therefore probably not caused by damage of the nerve, but might be ascribed to the arrest of a mechanism which accumulates potassium in the axon, and for which the required energy is lacking during asphyxiation.

SUMMARY

1. Isolated rabbit nerve kept in oxygenated Ringer's solution containing glucose, loses potassium during the first hours. No further loss is sustained during subsequent hours in this medium.

2. Asphyxiation even for the relatively short period of two hours caused a loss of potassium which may be ascribed to the arrest of a potassium accumulating process which requires energy.

I am indebted to Miss R. Estey who carried out the potassium determinations.

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THE EFFECT OF pH OF EXTERNAL SOLUTION ON OXYGEN CONSUMPTION OF ONION ROOT TISSUE

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TWO FIGURES

A great deal of work has been done on the effect of hydrogen ion concentration of external solution on oxygen consumption of plant tissues from which apparently varying results have been obtained. C. J. Lyon ('27) found that the respiration of *Elodea* increased with increasing alkalinity beyond pH 7.0. L. A. Thomas ('31) showed that respiration of carrot slices increased with acidity. Steward and Preston ('41) reported from a study of the effect of pH and phosphate concentration on the metabolism of potato discs that at constant phosphate concentration both respiration and protein synthesis were at a maximum at pH 7.0 and decreased in solutions more acid or alkaline. Bennet-Clark and Bexon ('43) in an extensive study of beet tissue metabolism immersed beet discs in a test medium of a plant extract with a pH of 3.0 (acidity due to malic and citric acids) and found that this extract caused a loss of pigment and a coincident fall in respiration rate. In an extract with a pH of 5.0 the respiration of the beet discs increased. Lundegardh and Burström ('44) concluded that the overall respiration of wheat seedling roots is independent of the pH of the external solution above pH 3.2. They gave no upper limit of pH above which respiration of the roots was lowered.

Hydrogen ion concentration apparently has an important effect, directly or indirectly, on the respiration of animal tissue also. The respiration of *Paramecium caudatum* was found to be optimum in solutions with pH's between 7.0 and 7.3 and had a wide tolerance on the acid side and a lower tolerance on the alkaline side. The respiration of the paramecium was very low in solutions of pH's between 3.0 and 4.0 and of pH's between 8.5 and 10.0 (Humphrey and Humphrey, '48). The respiration of sea urchin eggs was increased when hydroxyl ions were added to sea water (McClendon and Mitchell, '12). No pH values were measured. Hiestand ('42) found that the respiration of the sea cucumber, *Thyone briareus*, was inversely proportional to pH values of sea water between 5.4 and 8.8. The oxygen consumption of the larval form of a parasitic nematode, Eustrongylides, was unchanged in solutions of pH's between 3.4 and 8.3 but was increased between 1.1 and 2.0 and between 9.0 and 10.7, a somewhat unusual occurrence (von Brand, '43). Hopkins ('46) in his study of the marine clam, *Venus mercenaria*, found that the respiration of the gill tissue was 8% lower in sea water of pH 6.8 than in normal sea water with pH's 7.5 to 8.5. Humphrey ('46) found that muscle homogenates of the oyster had a respiration optimum in solutions with pH's around 7.4 and that it gradually decreased on either side of that pH optimum. Respiration of the whole barnacle, *Balanus crenatus*, was not changed in solutions with pH's of 7.2 to 8.2 (Kreps, '29). Respiration of boar and ram sperm declined as the pH was raised or lowered from an optimum between pH's 7.2 and 7.3. A 0.4 pH unit change was accompanied by significant lowering of respiration (Winchester and McKenzie, '41). The respiration of guinea pig kidney, brain, and liver tissues was constant in solutions of pH's 6.8 to 9.5. Oxygen consumption fell sharply to zero in solutions with pH's of 4.0 and below and of 11.0 and above. A decrease in respiration began in solutions of pH 9.5, and the effect was irreversible beyond that hydrogen ion concentration. However, reversibility on the acid side was obtained in solutions with a pH as low as 5.0 (Stearns, Greenblatt, Canzanelli, Rapport, '41).

Although there are differences in results from the variation of pH of external solution on the various plant and animal tissue respiration, lowered respiration at pH extremes occurred generally. In many of the above-mentioned studies, however, only one source of hydrogen ions was used, and most often only effects of either acidity or alkalinity were studied. In the experiments described below on onion root tissue at least two pH sources were used at each pH, and also the effect of both high and low pH's of external solution on oxygen consumption of excised onion root tissue was determined.

OXYGEN CONSUMPTION

Method. The Warburg direct manometric technique was used to measure oxygen consumption.

The onions were cultivated in aerated Hoagland solution in darkness for 72 hours. Ten straight, firm, 30 mm long apical root sections of approximately the same diameter were used as a sample in each Warburg flask of 10 to 11 ml capacity. The control solution covering the roots was aerated distilled water of pH 5.5 to 6.0. The test pH mixtures which were dumped at the end of a control period into the aerated distilled water and onion tips consisted of either hydrochloric acid or citric acid-Na_2HPO_4 buffer for pH's 2.2 to 8.0 or boric acid-KCl-NaOH buffer for pH's 8.0 to 9.6. A solution of Na_2HPO_4 was used also to make solutions of pH's 8.0 to 9.0. The molarities of these solutions were kept as low as possible (see table 1), since Steward and Preston ('41) found that a .002 M phosphate concentration caused an increase in potato slice respiration. The effect of phosphate concentration on respiration of onion roots was not determined in this study, but it may be noted that no difference in results occurred because of a difference in hydrogen ion source.

The 30 mm apical root sections were cut carefully under water and left in aerated distilled water for an hour before they were placed in Warburg flasks in order for them to become equilibrated to the environment. Twenty per cent KOH was placed in the center wells of the Warburg flasks to absorb the CO_2 given off by the roots. Subsequently flasks and contents were equilibrated in the constant temperature bath at 27.8°C. (± .1°) for and hour with the manometers open to the external atmosphere. After equilibration the manometers were closed, and a control period of at least an hour and a half ensued. Manometer readings were made at 10-minute intervals until oxygen consumption remained constant for an hour or longer. The test solutions were then dumped, and 10-minute readings were made again for at least an hour and a half.

TABLE 1

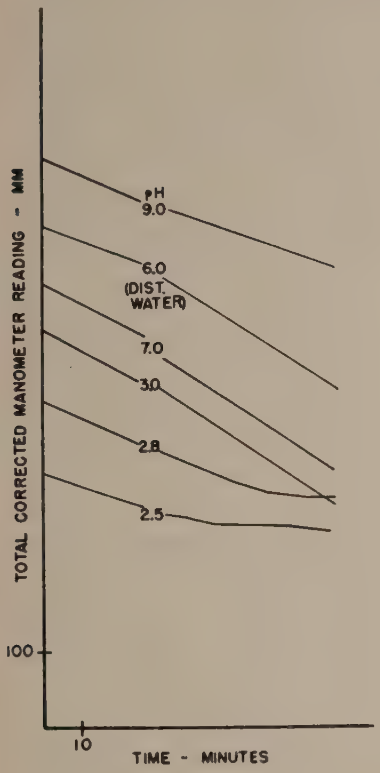
Molar concentrations of all compounds used as pH adjusters at the pH's used in all experiments

pH	MOLAR CONC. OF CITRIC ACID	MOLAR CONC. Na_2HPO_4
	Citric acid–Na_2HPO_4 buffer	
2.2	.00390	.00016
3.0	.00329	.00082
4.0	.00256	.00154
5.0	.00194	.00206
8.0	.00011	.00389

pH	MOLAR CONC. KCL	MOLAR CONC. BORIC ACID	MOLAR CONC. NaOH
	Boric acid–KCl–NaOH buffer		
8.0	.005	.005	.0002
9.0	.005	.005	.00107
9.6	.005	.005	.00193

pH	MOLAR CONC. HCl
	Hydrochloric acid concentrations
2.0	.01
3.0	.001
4.0	.0001
5.0	.00001

Results. The graphs which are shown in figures 1 and 2 are typical examples of results; at least 4 experiments with identical results were made at each test pH. Figure 1 indicates that a solution with a pH as low as 3.0 does not decrease or increase the rate of onion root respiration during the one and a half hour test period, regardless of pH source. However, a pH of 2.8 noticeably decreases oxygen uptake after



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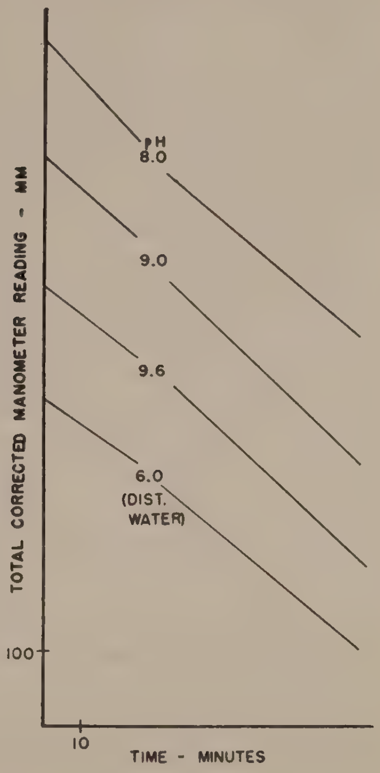

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Fig. 1 Effect of phosphate-citric acid buffer of various pH's on oxygen consumption of onion root sections. Oxygen consumption is indicated as total corrected manometer reading in millimeters of Brodie solution. The break in the curves indicates dumping of test solution into control mixture of distilled water of pH 6.0 and the onion roots.

Fig. 2 Effect of boric acid-KCl-NaOH buffer of various pH's on oxygen consumption of onion root sections. Oxygen consumption is indicated as total corrected manometer reading in millimeters of Brodie solution. Break in the curve indicates dumping of test solution into control mixture of distilled water of pH 6.0 and onion roots.

30 minutes. A solution with a pH of 2.5 causes immediate decrease in respiration of the onion root sections. It may be noted from figure 2 that there is no increase or decrease in respiration rate in solutions of pH's 8.0 to 9.6.

METHYLENE BLUE REDUCTION

Method. In connection with the problem of the effect of hydrogen ion concentration on onion root tissue respiration, relative rates of methylene blue reduction by root sections which had previously been immersed in solutions of various pH's were observed. The phenomenon had been noted by this writer and also by Norris ('48) that firm excised onion root sections consistently decolorized a 1–10,000 dilution of methylene blue of approximately the root section volume in one hour, and also that if the root had been poisoned or mechanically injured, the root would not reduce the dye to the leuco form at all or very slowly, depending on the degree of injury. Reduction of dyes by living cells is not, of course, a new observation. Cannan, Cohen, and Clark ('26) showed a general correlation between reduction potential of a cell suspension, cellular reduction of a dye, and reduction potential of the same dye as determined in pure solution. Earlier Michaelis ('02 on pp. 101 and 104) stated that many dyes such as methylene blue are reduced to colorless derivatives by certain cells while alive, but not when dead, so that reducing power in these cases may be used as a criterion of vitality.

Firm sister apical root sections of the same diameter and length were immersed in test pH solutions and some also in distilled aerated water for two hours. The latter were designated as control roots. At the end of that period the roots were washed singly with distilled water and placed in glass tubes of a slightly greater length and diameter than the roots themselves. The tubes had previously been filled with a 1–10,000 dilution of methylene blue. The ends of the tubes were sealed with paraffin, and the entire tube was sealed on a white glass plate. Observations and comparisons of the tubes were made with reference to a sealed tube containing methylene blue only and also with sealed tubes containing control roots and methylene blue. The observations were made at regular intervals for 5 hours. The temperature remained constant within ± 0.5°C. during the experiments.

Results. For complete details of the results table 2 may be consulted. Roots which had been immersed in solutions of pH 2.0 did not reduce methylene blue at all but were stained immediately by the dye and remained stained. Ten millimeters of the tips of roots which had been in solutions of pH 3.0 did not reduce methylene blue, although the remainder of the root did. Roots which had been in solutions of pH's 4.0 to 9.6 apparently were not injured, since they reduced methylene blue as fast as the control root did. The only observable injuries were on those roots which had been immersed in solutions of pH's 2.0 and 3.0. Rachaner ('42) found also by microscopic examination of onion root sections that injury, which was detected by shrinkage and translucency of the root, occurred in sulfuric acid solutions of pH's 2.0 and 3.0 and that no observable injury occurred at higher pH's.

DISCUSSION

It may be concluded that, under the conditions of the experiments, the range of pH of external solution over which the respiration of onion root sections is not affected is from pH 3.0 to 9.6. This information might be of value to those working on effects of poisons or stimulants on onion root respiration. As yet we do not know the effect of the various ions of the acids and buffers on onion root respiration. However, it is difficult to understand how the ions in the pH sources used in this work could affect the respiration rate in precisely the same way. Perhaps different results could have been obtained in certain cases if it had been feasible to run the experiments longer.

The apparently wide range of pH for favorable oxygen consumption may be due to the fact that the root itself probably has a stronger buffer system, so that the internal environment of the root is not affected to a large extent by the

TABLE 2

Relative rates of methylene blue reduction by excised onion roots which had been immersed in various pH solutions for 2 hours

TIME	pH 2.0	3.0	4.0	CONTROL 6.8	8.0	9.0	9.6
15 min.	Root blue. Dye soln. blue.	Apex of root blue. Basal end has begun to decolorize blue soln.	Apical 20 mm has decolorized dye around it.	Apical 20 mm has decolorized dye around it.	Same as control.	Same as control.	Same as control.
30 min.	Root and dye soln. same shade of blue.	Tip blue. Soln. same as control at pH 6.8.	Same as control.	Dye decolorized all along root to a faint blue.	Same as control.	Same as control.	Same as control.
1 hr.	Root and dye still dark blue.	Tip still blue. Soln. around root decolorized.	Same as control.	Dye almost completely decolorized.	Same as control.	Same as control.	Same as control.
1½ hr.	Root and dye still dark blue.	Tip blue. Adsorption of blue dye to rest of root begun.	Same as control.	Dye completely decolorized.	Same as control.	Same as control.	Same as control.
5 hr.	Root blue.	Root blue.	Same as control.	Dye still decolorized.	Same as control.	Same as control.	Same as control.

pH of the medium, as Lundegardh and Burström ('44) suggested from their work on wheat seedling roots. It may be noted again that they also found that the respiration of wheat roots was apparently independent of the external hydrogen ion concentration above pH 3.2.

The Warburg oxygen consumption results compare favorably with those of the methylene blue reduction experiments. No observable injury was found above pH 3.0 and no reduction in oxygen consumption occurred above the same pH.

SUMMARY

1. The effect of pH of external solution on oxygen consumption and methylene blue reduction of apical onion root sections was studied. Warburg direct manometric technique was used to measure oxygen consumption.

2. Onion root oxygen consumption was apparently not affected in solutions of pH's 3.0 to 9.6 over a one and one-half hour period. In solutions with pH's 2.5 and 2.8 root respiration was definitely decreased within an hour.

3. Methylene blue reduction rate by onion roots was not decreased after immersion of the roots in solutions with pH's 4.0 to 9.6 for two hours. Immersion for two hours in pH 2.0 solutions killed the entire root section, but immersion in pH 3.0 solutions injured only 10 mm of the root apex.

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SOME EFFECTS OF ULTRAVIOLET RADIATIONS ON THE METABOLISM OF CHLORELLA

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ELEVEN FIGURES

Presented herein are the results of a comparative study of the effects of ultraviolet radiation on metabolism in the green alga *Chlorella pyrenoidosa.* Similar studies have been reported on other microorganisms and limited data are available for Chlorella.

Giese ('41) reported that in *Achromobacter Fischeri* the division mechanism, luminescence, and glucose respiration were destroyed in that order with increasing doses of UV. For this and subsequent work the UV source was a Sterilamp which has about 80% of its output in the 2537 Å mercury line. Dosages which just prevented cell division had a delayed effect on glucose respiration which appeared only after several hours; with larger doses the effect was more severe and appeared earlier.

In yeast Giese ('42) observed a pronounced stimulation of the endogenous respiration in starved cells after UV irradiation. At the same time the rate of respiration of glucose was inhibited. A corresponding but less pronounced stimulation occurred in *Achromobacter Fischeri;* no stimulation was observed in sea urchin sperm, Tetrahymena, and Neurospora. In further work on yeast Giese and Swanson ('47) studied in greater detail the effects of UV irradiation on endogenous and exogenous respiration, fermentation, and carbohydrate storage. Fermentation showed a partial inhibition similar to that of glucose respiration. Storage of cellular carbohydrate during the respiration of glucose was found to be more severely inhibited than the rate of oxygen uptake. Experiments involving measurement of the RQ, effects of added yeast extract and various vitamins, and the use of other substrates failed to reveal any definite locus for the UV inhibition of exogenous respiration. The results were interpreted in terms of general enzyme destruction.

Arnold ('33a, b) observed that in *Chlorella pyrenoidosa* irradiation at 2537 Å inhibited photosynthesis but had only negligible effects on endogenous respiration. The surviving rate of photosynthesis decreased logarithmically with increasing time of exposure. The fraction of the surviving photosynthetic rate was the same when measured in continuous light at saturating intensities or in flashing light with long intervening dark periods. The differential effect of 2537 Å on photosynthesis and respiration was later used by Ruben, Kamen, and Hassid ('40) in their study of radiocarbon uptake. A dosage which gave 95% inhibition of photosynthesis had no effect on respiration, but also caused a 95% inhibition of dark uptake of C^*O_2. It was argued, therefore, that the dark uptake of CO_2 is an integral part of the photosynthetic rather than the respiratory system.

These recorded observations encouraged a further study of the effects of UV on Chlorella directed toward two objectives: (1) to obtain additional insight into the comparative physiology of UV effects, and (2) to examine the possibilities of UV irradiation as a tool for the separation of photosynthetic and respiratory processes in Chlorella.

After this investigation was well under way it was discovered that a parallel study was being made by Holt, Brooks, and Arnold ('51) in search for the locus of the UV effect on the photosynthetic mechanism. We have exchanged information with these workers and are grateful to them for a manuscript copy of their paper which will be treated as an earlier publication.

METHODS

Chlorella pyrenoidosa was grown under sterile conditions in a continuous-culture apparatus (Myers and Clark, '44) in

Knops solution at 25°C. illuminated at 90–100 foot-candles and aerated with 5% CO_2 in air. By photometric control the culture was diluted at just such a rate as to maintain it at one point on its growth curve. Cultures used for the present experiments were maintained at a population density in the range 2.5 to 3.0 mm^3 cells per milliliter. Highly uniform experimental material is provided by this method. Details of the apparatus, the nutrient media, and characteristics of the cells produced have been described previously (Myers, '46).

For experimental use the cells were harvested and a measured aliquot centrifuged out, washed twice in fresh Knops solution, and made up to volume in Knops. Iron, other microelements, and citrate used in the medium for growth were omitted in the Knops used as a suspending fluid for irradiation and manometric experiments. This procedure originated as a convenience but later proved to be of importance in maintaining a fluid of high UV transmission. As measured by a Beckman Spectrophotometer, a 1.0 cm quartz cell has transmissions at 2537 Å as follows for the following fluids: water, 0.86; 0.1 M $KHCO_3$, 0.85; Knops, 0.80; Knops + 13.3×10^{-5} M ferric iron + 56×10^{-5} M citrate, 0.24. In order to obtain uniform cell suspensions it proved necessary to wash the cells free of iron citrate from the growth medium.

Cell suspensions were exposed to UV in a fused quartz absorption cell of 1.00 cm thickness and 5 cm diameter with two openings. The suspension was agitated and aerated by a stream of 5% CO_2 in air throughout irradiation. Exposure was terminated by withdrawing an aliquot of suspension by pipette and transferring it to a volumetric flask or directly to a Warburg vessel.

Germicidal lamps were used as a source of UV. About 80% of the radiation is in the 2537 Å line and the silica glass envelope cuts out most of the shorter wave length radiation. Two different set-ups were employed. The first utilized two small 4-watt GE lamps placed on opposite sides of the absorption cell; it was discarded because of the rapid decay characteristic of the output of these lamps. The second apparatus utilized a single 15-watt GE lamp, operated from a constant voltage transformer and cooled to approximately constant temperature by a forced current of air. The lamp was enclosed in one compartment of a box having a 6 inch square Corning no. 791 filter as a window. The absorption cell was held in another compartment 6 cm from the lamp and 4 cm from the filter. The total intensity at the absorption cell was 1100 microwatts/cm^2 as measured by a Moll large-surface thermopile which had been calibrated against a Bureau of Standards lamp.

Intensity of irradiation was controlled by insertion of one or more copper screens. Transmission of the screens was measured by a barrier-type photocell (Electrocell) and microammeter with the reasonable assumption that screen transmission is independent of wavelength from 2537 to 5000 Å. Transmissions were as follows: 1 screen, 70%; 2 screens, 49%; 4 screens, 23%. Because of the necessity of using suspensions of various density in the absorption cell no attempt was made to calculate dosage in absolute units. It was convenient to designate intensities in the following relative units:

INTENSITY DESIGNATION	NUMBER OF SCREENS	INCIDENT INTENSITY ON ABSORPTION CELL
2I	0	1100 microwatts/cm^2
I	2	550
I/2	4	275

Photosynthetic and respiratory activities of the cells were determined by standard manometric methods. Photosynthesis measurements were made in rectangular Warburg vessels in a bath with a glass bottom thermostated at 25°C. and illuminated from below by a bank of 17 60-watt lamps. Respiratory gas exchange was measured in conical Warburg vessels in a completely darkened bath also at 25°C. Inhibition of cell division was studied by a specially designed method which is described below.

RESULTS

1. Cell division and growth. Effects of UV on growth were first examined by plating methods. Repeated trials with variations in media and other details gave inconsistent results. Inhibition of colony formation by UV could be shown qualitatively, but within no one experiment were the quantitative data internally consistent. For Chlorella the method is complicated by the long incubation period of several weeks needed to develop visible colonies.

It appeared possible to observe the effects of UV on growth by a study of the growth curve. At saturating light intensity *C. pyrenoidosa* grows at a constant rate of about 0.85 log units per day. If a series of dilutions of a cell suspension are started at a given time, then, after a growth period, the relative concentration of the initial suspensions can be calculated from the separation of the log curves. If, instead of diluting, different fractions had been killed by UV, the amount of separation of the log curves should reveal the fraction killed. The method should be particularly suitable for Chlorella which is not stimulated by any organic substrate or growth substance during photosynthetic growth at light saturation. Living cells are, therefore, not expected to show any response to materials liberated by the killed cells.

The method was applied by following growth in test tubes containing 10 ml of suspension in Knops solution, aerated with 5% CO_2, and immersed in a 25°C. bath illuminated at about 600 f.c. by a bank of fluorescent lamps. Growth was determined in terms of optical density in an Evelyn photoelectric colorimeter with 600 mμ filter. Figure 1 shows growth curves (log density against time) for three dilutions of a Chlorella suspension freshly harvested from the continuous culture apparatus and one sample which had been irradiated. There is uncertainty in the lower portions of the curves attributable to transition phenomena accompanying the change to light saturation from the light-limiting conditions in the continuous-apparatus. However, in the upper portions of the curves the displacement of the curves corresponds very closely to that expected from the original dilutions. Table 1 presents the data and calculations of one experiment. In figure 2 the calculated values (4th column, table 1) have been plotted against the actual values corresponding to the original dilutions (first

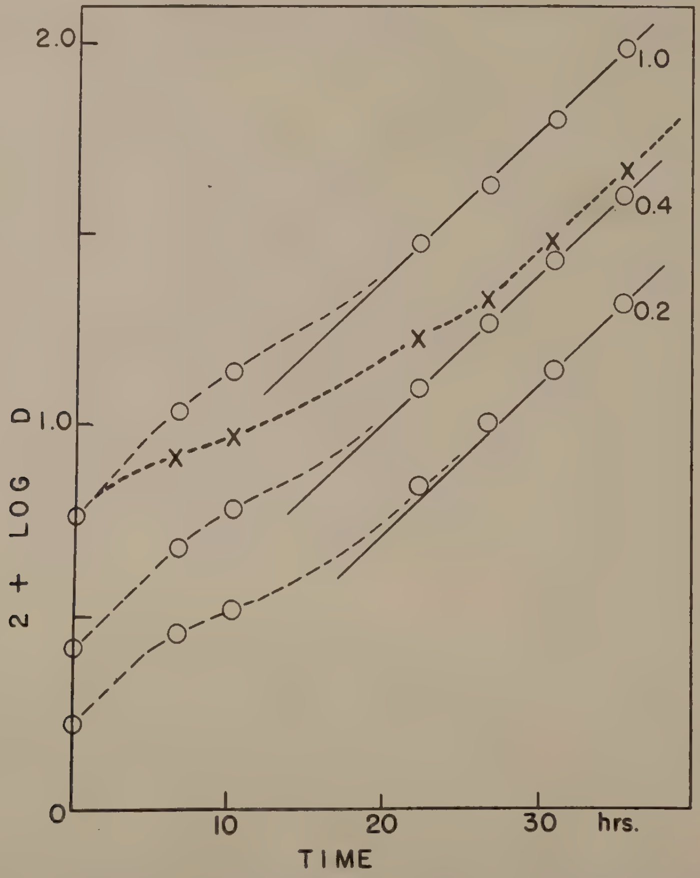


Fig. 1 Illustration of the method used to estimate UV inhibition of growth. Cell quantity measured in terms of optical density, D. Growth curves indicated (o o o o) describe untreated cells from a suspension containing 12 mm³ cells/ml in dilutions of 1.0 ml, 0.4 ml, and 0.2 ml to 10.0 ml. Growth curve (x x x x) describes 1.0 ml of the same suspension irradiated 10 minutes at intensity I and diluted to 10.0 ml.

column, table 1) for two separate experiments. Figure 2 thus becomes a calibration curve for the method.

For the UV killing experiments cells were irradiated in the same density of suspension (12 mm^3/ml) subsequently used for the respiration experiments. Aliquots of 1.0 ml were withdrawn at various times and diluted 1:100 to give the inocula for the growth experiment. The killed cells as well as living cells contribute to the initial optical density, but as growth of the living cells proceeds the effect of the killed cells becomes progressively smaller and finally negligible. After 24 hours the family of growth curves obtained by varying UV dosages and degrees of killing is strikingly similar to the corresponding family of curves obtained by making known dilutions (fig. 1). The various displacements between the log curves for irradiated cells were read off and the fraction of survivors calculated for each irradiation time (table 2).

TABLE 1

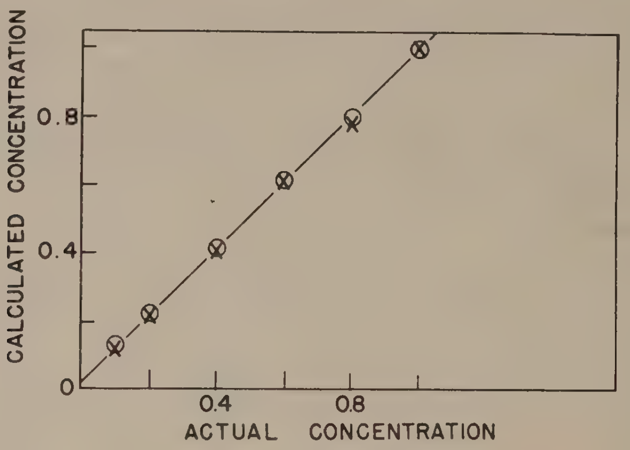
DILUTION FACTOR	LOG D AT 35 HRS.	DISPLACEMENT AT 35 HRS.	CALCULATED CONC.
1.00	1.985	0.000	1.00
0.80	1.877	— 0.108	0.78
0.60	1.770	— 0.215	0.61
0.40	1.595	— 0.390	0.406
0.20	1.317	— 0.668	0.214
0.10	1.049	— 0.936	0.116

The corrected concentrations (5th column, table 2), determined according to figure 2, represent the fraction of survivors of each irradiation time.

Effects of UV on cell proliferation are presented in figure 3 as a plot of logarithm of survival ratio against time of irradiation. The logarithmic character of the killing curve is typical of that observed for killing of other microorganisms by physical agents.

2. Endogenous respiration. In Chlorella, as in other microorganisms, two types of respiration may be distinguished.

In the absence of added substrate the cells show a low and nearly constant rate of oxygen uptake which, after a period of several hours, decreases to a still lower value. During this time reserve carbohydrate, identifiable as starch, gradually disappears (Cramer and Myers, '49). This may be termed the endogenous respiration. When a utilizable sub-



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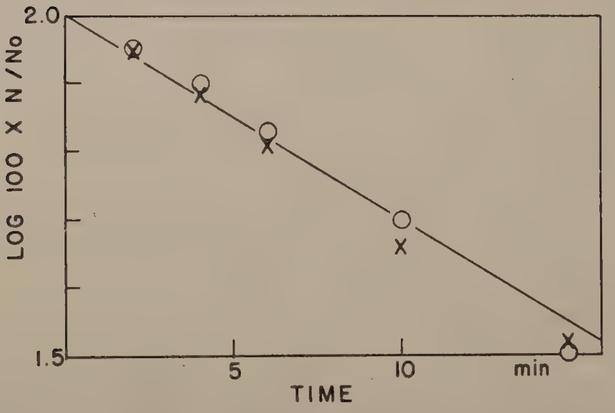

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Fig. 2 Calibration of the growth-curve method. Vertical displacements between the curves (cf. fig. 1) at 35 hours were used to determine the "calculated concentrations" of the ordinate. "Actual concentrations" are those obtained from the original dilution factors. The points represent two experiments; points indicated (x x x) are taken from table 1.

Fig. 3 Effect on growth of UV irradiation at intensity I.

strate such as glucose is added, the rate of oxygen consumption increases several fold to a final constant rate. This is the exogenous respiration which in the present discussion will be referred to more specifically as the glucose respiration.

Figure 4 presents the results of a typical experiment which shows the characteristic response of the endogenous respiration to UV irradiation and also allows comparison of effects of UV on the endogenous and glucose respiration. Curve A describes the oxygen uptake of normal cells in endogenous respiration. Curve E describes the transition from endogenous to glucose respiration when 0.02 molar glucose was added at the time indicated. After 10 minutes of UV irradiation, curve B, there is no effect on the endogenous respiration but the cells fail completely to respond to added glucose. After a 30 minute irradiation period, curve C, there is an initial increase in rate but the cells return to the normal endogenous rate after about 60 minutes; identical behavior is shown in curve D even though glucose is added at the indicated time.

TABLE 2

EXPOSURE	LOG D AT 35 HRS.	DISPLACEMENT AT 35 HRS.	CALCULATED CONC.	CORRECTED CONC.
min.				
0	1.987	0.00	1.000	1.00
2	1.935	— 0.052	0.886	0.89
4	1.890	— 0.097	0.796	0.80
6	1.818	— 0.169	0.675	0.67
10	1.670	— 0.317	0.482	0.48
15	1.520	— 0.467	0.344	0.33

The data of figure 4 and all other experiments allow a simple general statement. Except for a temporary stimulation of short duration, the endogenous respiration shows no response to a UV dosage which severely inhibits the glucose respiration and significantly inhibits photosynthesis. These results confirm those of Arnold ('33a) who also found no significant effect on the endogenous respiration. The transitory stimulation of endogenous respiration is qualitatively similar to the effect observed by Giese ('42) in yeast. Quantitatively, however, the changes are so small that no systematic study of effects on endogenous respiration has been made.

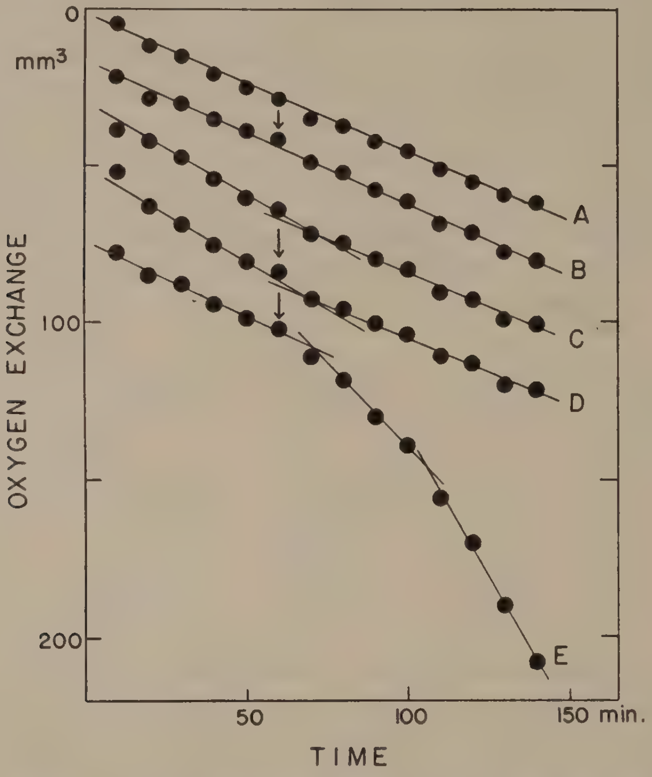

Fig. 4 Effects of UV on respiration. Curves A and C describe endogenous respiration. Glucose was added to B, D, and E at the time indicated by the arrows. Curves A and E, untreated cells; curve B, 10 minutes' UV; curves C and D, 30 minutes' UV. Cell suspension containing 14.4 mm^3 cells/ml was irradiated by two 6-watt germicidal lamps each 6 cm from the absorption cell; 2 ml aliquots were pipetted directly to conical Warburg vessels for measurements.

3. Glucose respiration. In contrast to the endogenous respiration the respiration of glucose is readily inhibited by UV. The normal response of Chlorella to added glucose is shown

by curve E of figure 4. Cells which have been irradiated at long exposures show no response to added glucose (curves B and D of fig. 4). With smaller dosages partial inhibition can be achieved.

A number of characteristics of the effects of UV on glucose respiration are summarized in table 3. The RQ is not appreciably altered (exp. A). The high value of the RQ is a consequence of accompanying nitrate reduction (Cramer and Myers, '48). Lack of response of the RQ therefore indicates that the ratio of carbon and nitrogen assimilation remains constant in spite of severe inhibition of the over-all process of glucose respiration. The pH and carbon dioxide concentration during irradiation do not seriously affect the extent of UV inhibition (exp. B). Acetate is the only other substrate readily respired by Chlorella but it is utilized only at higher pH; at pH 4.5 acetic and other fatty acids are highly toxic. The respiration rate showed a somewhat greater

TABLE 3

Effects of UV on exogenous respiration

EXPT.	DETAILS		100 X R/R$_0$	
A	No UV	RQ 1.46	100	
	6 min. UV	RQ 1.43, 1.45	58	
B	pH 4.5, aerate 5% CO_2	equal irradiations of 2 min. and 5 min. UV	66	40
	pH 6.8, aerate air		67	32
C	Substrate acetate	pH 6.8; equal irrad. of 3 min. and 7 min. UV	52	35
	Substrate glucose		37	25
D	2.0 ml suspension irradiated		30	$\times \frac{1}{2} = 15$
	2.0 ml suspension not irradiated		100	$\times \frac{1}{2} = 50$
	1.0 ml irradiated + 1.0 ml not irradiated		68	65
E	2 UV|1.5 dark|2 UV	4 min. UV	70	
	4 UV		70	
	2 UV|1.5 dk|2 UV|1.5 dk|2 UV|1.5 dk|2 UV	8 min. UV	48	
	4 UV|1.5 dk|4 UV		44	
	8 UV		44	

survival ratio on acetate than on glucose (exp. C). However, further inspection of the data showed that while the uninhibited control rate of oxygen uptake was greater with glucose (4.8 mm^3 O_2/hour/mm^3 cells) than for acetate (3.2), after irradiation the rates were about the same (1.8 on glucose, 1.7 on acetate after 3 minutes' UV; and 1.0 on glucose, 1.15 on acetate after 7 minutes' UV). Since it appeared that acetate and glucose respiration were inhibited by the same mechanism no further studies with acetate were made. The effect on glucose respiration is due to a direct action of UV on the cells and not an indirect effect of action on the medium (exp. D). A given UV irradiation has the same effect whether applied in a solid exposure or in shorter periods interrupted by dark periods (exp. E). It should also be noted that with glucose respiration there is no delayed effect or other transitory phenomenon; the surviving rate of oxygen uptake remains constant for at least 4 hours after irradiation.

The relation of survival ratio of glucose respiration to UV dosage has been studied by varying both the intensity and duration of irradiation. Figure 5 presents the results of a series of 7 experiments as a plot of the relative rate of oxygen uptake against dosage. The rate drops off very rapidly at first and then gradually approaches the endogenous rate with increasing dosages. (Data on the endogenous rate are included in a box at the end of the curve since they really do not belong anywhere within the figure.)

The data of figure 5 and the previous observations in figure 4 suggest a simple hypothesis. During glucose respiration the rate of oxygen uptake is decreased logarithmically with increasing dosage, but only down to the level of the endogenous rate. The curve of figure 5 has been drawn in according to the equation $\log (R-23) = \log (100-23) - 0.11\,D$ where (R-23) is the observed rate minus the endogenous rate and D is the relative dosage. The curve provides sufficient fit to the data to justify the hypothesis, at least as a close approximation.

Attention should be called to the fact that even with a 4-fold variation in intensity of irradiation the data fit a common dosage curve. This demonstrates that the intensity X time relation holds for this phenomenon since the relative dosage was calculated as the product of relative intensity and time of irradiation.

Holt, Brooks, and Arnold ('51), using the same strain of Chlorella, observed a less regular decay of the glucose respiration with increasing dosage and interpreted their results in terms of two types of Chlorella cells or two separately-inactivated processes of glucose respiration. We have searched in vain for some simple explanation of this difference in behavior observed in the two laboratories. We have observed that in starved cells there appears to be a lower sensitivity to UV irradiation. If the cell preparation were not reasonably homogeneous (i.e., if it contained a mixture of growing

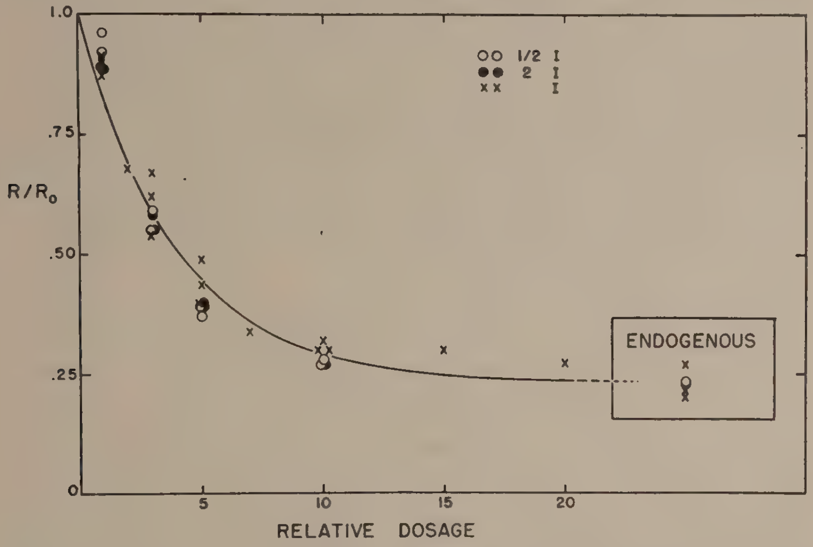

Fig. 5 Effect of UV on glucose respiration. Relative dosage has dimensions of minutes × intensity. A complete plot of 7 experiments. Irradiated suspensions in Knops solution contained 13 to 13.5 mm^3 cells/ml; 2 ml aliquots were transferred directly to Warburg vessels.

and starved cells), then the dosage curve would reflect the cell variation.

4. Photosynthesis. Following a dose of UV radiation which causes partial inhibition of photosynthesis the rate of gas exchange remains constant for a period of about 30 to 50 minutes. After longer periods of photosynthesis the rate gradually declines. This behavior is the same whether measured in Warburg no. 11 carbonate-bicarbonate buffer or in Knops solution at pH 4.5 saturated with 5% CO_2. It was shown by the two-vessel method that the normal CO_2/O_2 quotient of 0.90 is not changed by irradiation. Most of the measurements of photosynthesis have been made in Knops solution saturated with 5% CO_2 and oxygen evolution calculated by assuming a quotient of 0.90. By experiments similar to that of table 3D it was established that inhibition of photosynthesis is caused by direct action on the cells and not by an indirect effect of irradiation of the medium.

Survival curves for the initial rate of photosynthesis as a function of dosage at different intensities are presented in figure 6. As the UV dosage is increased the subsequent initial rate of photosynthesis falls off, at first logarithmically and then more rapidly than the logarithmic decay expected of a first-order process. The break-away from logarithmic behavior is more severe after higher intensities of irradiation at which lower survival ratios were attained. This behavior was not anticipated from the original results of Arnold ('33a) which, however, are far too scanty to afford precise comparison. Holt, Brooks and Arnold ('51) also report a negative curvature for their logarithmic plot of photosynthesis inhibition for both Chlorella and Scenedesmus.

The present data, of extended usefulness because of the range of intensities used, indicate that the inhibition of photosynthesis by UV begins as a first-order process; a secondary process, apparenty of higher order, becomes operative as the dosage is increased and more severely so at higher intensities. With the idea that such a secondary process appears to be rate-dependent we have explored the possibilities of varying the rate of metabolic processes occurring during irradiation. Starvation of the cells before irradiation, addition of glucose before irradiation, or illumination with a photosynthesis-saturating intensity of visible light during irradiation, all failed to cause appreciable change in the degree of inhibition of photosynthesis caused by high-intensity UV irradiation. No explanation of the intensity-dependent nature of the UV inhibition of photosynthesis is now available.

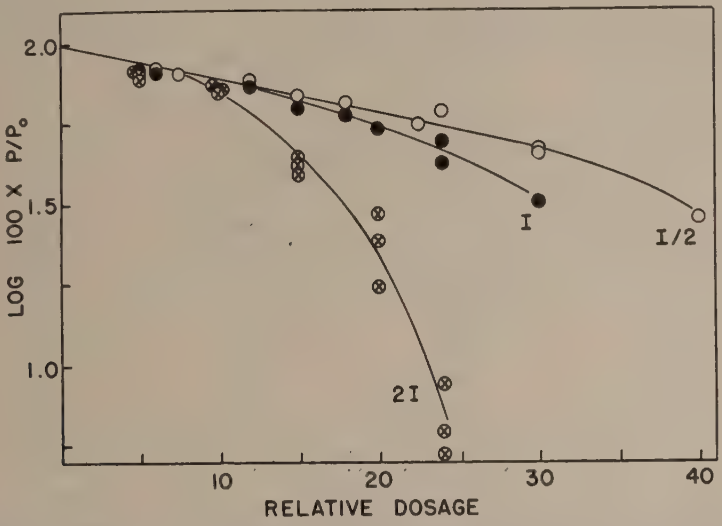

Fig. 6 Effect of UV on the following intial rate of photosynthesis. Relative dosage has dimensions of minutes × intensity. Irradiated suspensions in Knops solution contained 1.75 to 1.90 mm^3 cells/ml; 2 ml aliquots plus 4 ml fresh Knops solution were placed in rectangular Warburg vessels and gassed with 5% CO_2 in air. Light intensity for photosynthesis measurement was 1000 f.c.

The survival ratio of photosynthesis as a function of light intensity has been studied by determining the light intensity curves for normal and irradiated cells. Table 4 summarizes one experiment illustrating the general result that the survival ratio of photosynthesis is independent of the light intensity used in measurement. This corresponds to the observation of Arnold ('33b) that inhibition is the same whether measured in saturating continuous light or in flashing light.

By classical argument, therefore, locus of the UV effect is neither specifically photochemical nor specifically enzymatic. Alternately one anticipates a unit-by-unit type of inactivation. However, the response of the photosynthetic mechanism to UV irradiation now appears far too complex to argue for a photosynthetic unit in the classical sense (cf. Arnold, '33b).

The rate of photosynthesis which follows a UV irradiation does not remain constant but slowly decreases to a still lower rate. Curve B of figure 7 represents such a time course of photosynthesis in cells which had been irradiated to give an initial 50% inhibition.

TABLE 4

Survival ratio of photosynthesis (initial rate) as measured at different light intensities in Warburg no. 11 buffer

LIGHT INTENSITY	CONTROL	7½ MIN. IRRADIATION	SURVIVAL RATIO
foot-candles	*mm^{3}O$_2$/hr./mm^3*	*mm^{3}O$_2$/hr./mm^3*	
75	9.8	3.0	0.30
255	24.5	6.7	0.27
650	32.5	9.3	0.28
1100	33.0	9.6	0.29

This behavior submits to the following explanation. A fraction of the total photosynthetic capacity, not at all affected by the UV irradiation, contributes to the total oxygen evolution according to the extrapolated curve B′. Curve B approaches B′ as there is used up some substance, *S*, required for photosynthesis and initially present in abundance. The decay in concentration of *S* may be obtained by point-by-point subtraction of curve B from curve B′; the decay approaches that expected of a first-order reaction. As a second approximation it is recognized that curve B really is approaching a final rate somewhat lower than that of B′ and not yet attained within the time of the experiment (cf. fig. 8, below). If the final rate is arbitrarily located by the extrapolated curve B″ then the predicted decay in concentration of *S* is found to follow closely the expectations of a first-order reaction.

A question now arises whether the decay in *S* occurs only during photosynthesis or whether it also may occur independently. In the experiment of figure 7 equal aliquots of irradiated cells were placed in each of 4 Warburg flasks. Flasks A and B were illuminated at a time 20 minutes after the beginning of the UV irradiation. Flasks C and D were shaken in a darkened bath and placed in the light after one or two hour intervals. Rates of oxygen evolution, determined from tangents to the curves, are indicated by the figures along the curves. If these figures on the three curves are compared horizontally they show good agreement, if compared vertically they show wide variation. The concentration of *S* surviving a given UV dosage is, therefore,

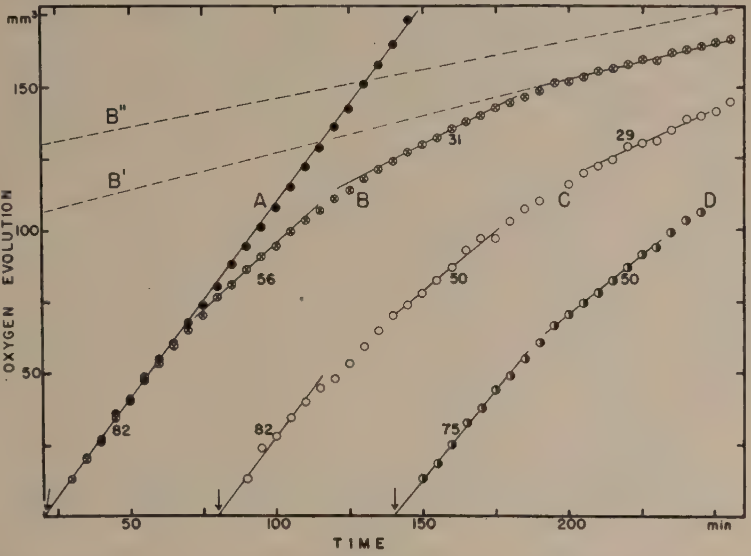

Fig. 7 The time course of photosynthesis following UV irradiation. The time axis indicates minutes after the beginning of irradiation. Cell suspension contained 1.6 mm^3 cells/ml in Knops solution; 1 ml aliquot plus 5 ml Knops were transferred to vessel A; after 7.5 minutes' irradiation at intensity 2I, 2 ml aliquots plus 4 ml Knops were transferred to vessels B, C, D. Vessels were gassed with 5% CO_2 in air, shaken in dark, and transferred to 1000 f.c. illumination at the times indicated by the arrows.

a function of the amount of photosynthesis and relatively independent of total time after irradiation.

Further information has been obtained by following the course of photosynthesis in aliquots of cells which had received varying doses of UV. One such experiment is shown in figure 8. It is apparent that the survival ratio of photosynthesis measured after 5 hours is considerably less than that observed immediately after irradiation. From the experiment of figure 8 and another companion experiment the survival ratios have been determined in terms of the rates during each hour of photosynthesis following the UV irradiation. The combined results of the two experiments are presented in figure 9 as a semilog plot of survival ratio against relative dosage. The upper curve describes the survival ratio in terms of the rate of photosynthesis over the first hour of illumination; the lower curve describes the survival ratio in terms of the 5th hour of photosynthesis. Intermediate points

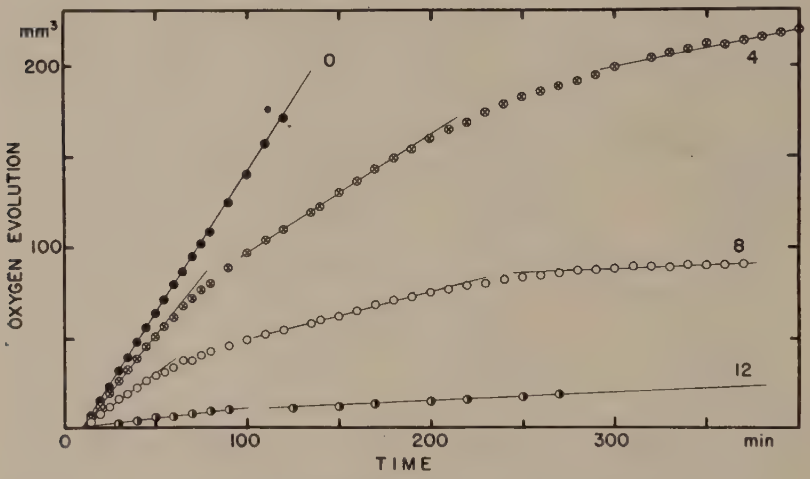

Fig. 8 The time course of photosynthesis after various dosages of UV. The time axis indicates minutes from the beginning of illumination at 1000 f. c. A cell suspension containing 1.8 mm³ cells/ml was irradiated at intensity 2I; a 1 ml aliquot was transferred to each vessel with 5 ml of Knops after the minutes of UV indicated by the figures on the curves.

for the 2nd, 3rd and 4th hour show considerable scatter, but appear to establish a series of curves intermediate between and roughly parallel to those for the first and 5th hours.

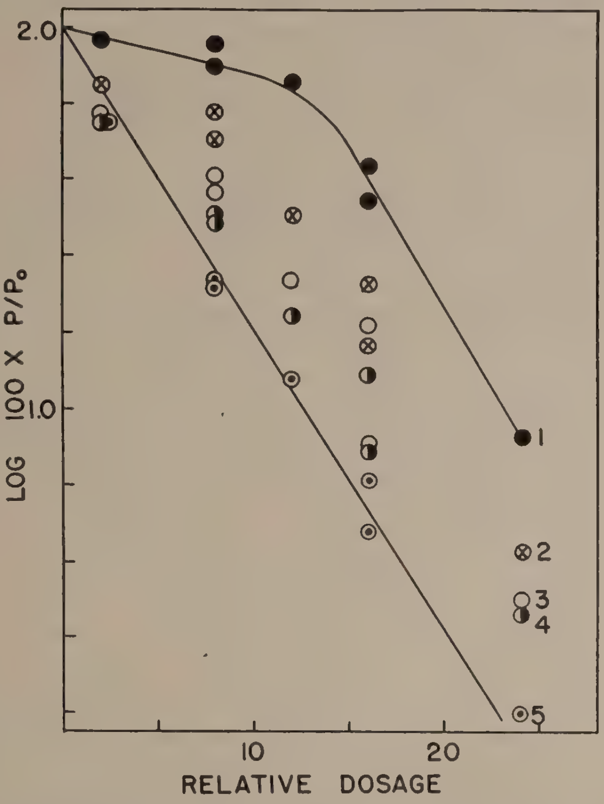

Fig. 9 Survival curves for photosynthesis as measured at various periods following irradiation. The data are taken from the curves of figure 8 and another companion experiment. Survival ratios are calculated for the rates of photosynthesis over the hourly intervals indicated for the various points at the lower right.

The series of curves of figure 6 and figure 9 lead to the conclusion that at least two components within the photosynthetic mechanism are inactivated by UV. The less sensitive component is inactivated according to the upper curve of figure 6 and the upper curve of figure 9; this inhibition is evidenced in the initial rate of photosynthesis. Inactivation of the more sensitive component proceeds along a dosage curve approximated by the lower curve of figure 9; the extent of this second type of inhibition is completely apparent only after an extended period of photosynthesis and the attendant removal of the substance *S*.

The lower curve of figure 9 is drawn with some uncertainty. After still longer periods of photosynthesis than those studied in the experiments of figure 8, the rate may decline further, giving rise to still lower survival ratios and an inhibition curve displaced downward from the lower curve of figure 9. A related qualitative observation is that at the end of experiments such as those of figure 8 the cells appeared bleached, the effect becoming progressively more severe with the length of the preceding UV irradiation. This effect is qualitatively similar to that noted by Myers and Burr ('40) as a result of exposure to very high intensities ($\sim$ 30,000 f.c.) of visible light. It is possible that after UV irradiation even the intensity of illumination ($\sim$ 1,000 f.c.) used here may cause an inhibition of the photosynthetic apparatus superimposed upon the UV effect.

5. Comparison. It appeared desirable to compare the extent of inhibition as a function of UV dosage for each of the processes studied. With our facilities such comparison demanded constant cell density in the irradiation absorption cell as well as constant incident intensity for the different experiments. The cell density used was determined by the requirement of about 12 mm^3 of cells per milliliter for adequate measure of respiration. In contrast, a population of 1.0 mm^3/ml suffices for photosynthesis measurements. Additional experiments were performed on photosynthesis inhibition, using the same high density in the absorption cell as in the respiration experiments and diluting immediately after irradiation to obtain the desired low density of cells for the photosynthesis measurements. Similar procedure had already been applied in the experiments on growth inhibition (cf. fig. 3).

Comparable dosage-inhibition curves for 4 cellular processes are presented in figure 10. To a good approximation the data can be fitted to straight lines, indicating in each case a simple logarithmic decay. The relative sensitivity to UV of the 4 processes, calculated from the slopes of the curves and from the dosage to give 50% inhibition, is summarized in table 5.

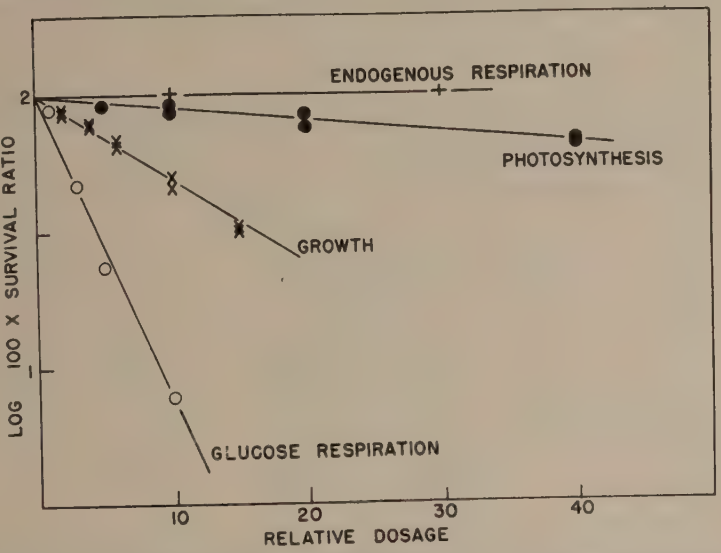

Fig. 10 Comparison of the sensitivities to UV of 4 cellular processes. Relative dosage has dimensions of minutes × intensity. Cell suspensions in Knops solution were irradiated at a density of 12 to 13.5 mm^3 cell/ml. The curves for growth and glucose respiration were taken from figures 3 and 5. The curve for photosynthesis describes the initial rates observed in special experiments with dense suspensions.

Because of greater uncertainty in its determination the inhibition of the final rate of photosynthesis is not included in figure 10. Two experiments similar to those of figure 9, but based on irradiation of the same dense suspensions used in the comparison experiments, do afford comparative data and are summarized in figure 11. The upper series of points, determined from the initial rates of photosynthesis, are fitted by a curve of the same slope as that in figure 10. The lower solid points indicate the survival ratios for the ''final'' rates of photosynthesis which in this case were determined over the period of the 4th hour of illumination. For comparison there has been drawn in the survival curve for growth. It appears that the UV inhibition, as measured by the rate of photosynthesis after 4 hours of illumination, is approximately the same as that measured by the capacity of the cells to grow under photosynthetic conditions.

DISCUSSION

The high uniformity of experimental material has allowed quantitative comparison of the effects of UV irradiation on different metabolic processes in Chlorella. Of those studied, glucose respiration is by far the most sensitive. Chlorella has two initial alternative pathways of carbon synthesis which will support growth, one from exogenous substrate such as acetate or glucose by oxidative assimilation, the other from carbon dioxide by photosynthesis. The comparison curves of figure 10 indicate that in an irradiated population the ability to utilize glucose (or acetate) may be almost completely destroyed even though a considerable fraction of the population retains the ability to photosynthesize and to perform all the complex biosyntheses required for the formation of new cells. UV irradiation, therefore, must affect some early stage in glucose assimilation not otherwise required for cell metabolism. Because of the very high sensitivity one surmises that

TABLE 5

Comparative sensitivity to UV irradiation

	FROM SLOPE OF SURVIVAL CURVE		FROM MINUTES TO GIVE 50% INHIBITION	
	Slope	Sensitivity	Minutes	Sensitivity
Endogenous respiration	0	0	(∞)	0
Photosynthesis	0.0045	1/25	66	1/22
Growth	0.030	1/3.7	10	1/3.3
Glucose respiration	0.11	1.0	3	1.0

the physical site of this inhibition lies on or near the cell surface.

Peacocke and Hinshelwood ('48) have observed what may well be a related case of inhibition of glucose utilization by UV irradiation. From a UV-irradiated suspension of *Bact. lactis aerogenes* they obtained a number of different strains by single cell isolations. Certain of these strains showed a long lag in growth on glucose and ammonium salt medium as compared to a very short lag on asparagine-glutamate medium; the lag was reduced also by oxalactate and α-ketoglutarate. From this and related evidence it was postulated that the site of UV damage occurred in an enzyme required in the breakdown of glucose to keto-acids.

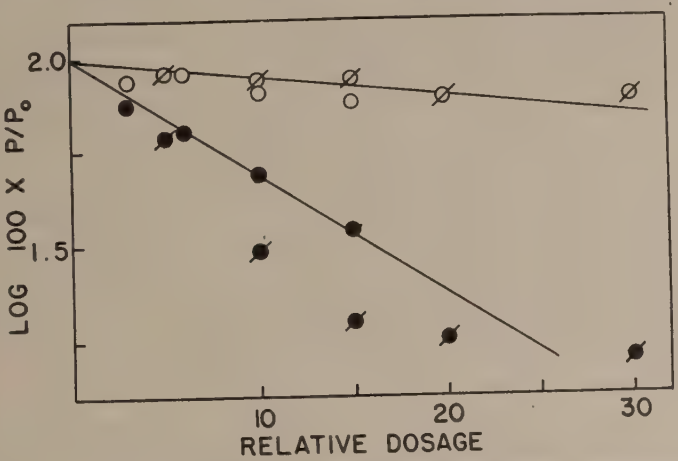

Fig. 11 Comparative sensitivity to UV of the "final rate" of photosynthesis. Cell suspensions for irradiation contained 12.5 mm^3 cells/ml. Open points indicate survival of photosynthesis in terms of the initial rate; solid points are in terms of the rate during the 4th hour of illumination. The upper line is drawn in with the same slope as the "photosynthesis" curve of figure 10; the lower curve reproduces the curve for "growth" of figure 10.

It is noteworthy that the endogenous and exogenous respiration of Chlorella respond to UV irradiation in the same way in which they respond to cyanide inhibition (Emerson, '27). It thus appears likely that UV and cyanide affect a common cellular component required for glucose respiration. In studies on oxidative assimilation (e.g., Barker, '36) it has been held, although with some uncertainty, that the endogenous respiration is suppressed during assimilation of an easily oxidizable substrate. Unfortunately the inhibition by UV offers no apparent advantage over cyanide in clarifying relationships between the two processes.

If the parallel between UV and cyanide inhibition observed for respiration is extended to photosynthesis, then it is to be expected that inactivation may occur in several different enzyme systems. In *Chlorella pyrenoidosa* severe inhibition of photosynthesis is induced by cyanide concentrations which have negligible effect on endogenous respiration (Emerson, '29). In other algae quite different effects have been reported. These, and other observations on cyanide effects, have led Rabinowitch ('45, p. 309) to postulate inhibition of several enzymes. Specifically suggested are a carboxylating enzyme, E_A, and oxygen-liberating enzyme(s), E_O.

Data on UV inhibition also give direct support to the idea that several different systems may be affected. Reference has been made above to the observation of Ruben, Kamen, and Hassid ('40) that UV reduces the capacity for radioactive CO_2 uptake in the dark as well as in the light. This effect may be interpreted in terms of a carboxylating enzyme, E_A. Holt, Brooks, and Arnold ('51) report equal sensitivity to UV of the Hill Reaction (oxygen evolution from an oxidant such as ferric iron substituted for carbon dioxide) and photosynthesis in Scenedesmus. Here the oxygen-liberating enzyme, E_O, may well be the locus of inhibition.

Examination of the time course of photosynthesis following a UV irradiation provides additional description of the delayed action effects noted by Holt, Brooks, and Arnold ('51). The initial rate of photosynthesis approaches a lower final rate achieved only after a period of several hours. The simplest possible explanation requires inactivation of two different enzymes of the photosynthetic mechanism. The second enzyme, which determines the final rate, is more sensitive to UV but the effects of its inactivation are initially obscured by a utilizable substance, *S*. As photosynthesis proceeds, *S* is used up until the over-all rate becomes dependent on the concentration of the second enzyme. However, further consideration reveals difficulties in this simple explanation. The amount of *S*, or the ability to utilize it, also decreases with increasing UV dosage as may be seen by inspection of the curves of figure 8. Further, *S* is required in very high initial concentration. In figure 7 the amount of *S* indicated by the extrapolated intercept corresponds to an evolution of about 6 micromols of oxygen from 3.23 μl of cells. An initial cellular concentration of nearly two molar in terms of O_2 equivalents would be required.

In search of explanation of the present results we have considered and abandoned a number of alternative hypotheses based upon inactivation of two different enzyme systems and participation of the substance, *S*. More complex schemes might be considered but it is doubtful that they would serve a real purpose in suggesting more critical experiments with Chlorella. A more fruitful line of attack appears to lie in the study of sub-cellular preparations or in the use of algae less limited by permeability restrictions in their utilization of possible metabolic intermediates. Such an attempt has been made by Holt, Brooks, and Arnold ('51) on chloroplast preparations, but without success in defining the locus of UV inhibition.

The expectation that UV irradiation might provide a simple separation of photosynthetic and respiratory processes is not borne out by the present results. Rather, there are several different types of inhibition, three of which are demonstrated in Chlorella: one affects an early step in exogenous respiration and at least two others are located in the photosynthetic mechanism.

SUMMARY

1. Effects of ultraviolet irradiation (principally 2537 Å) on *Chlorella pyrenoidosa* have been studied in terms of growth,

endogenous respiration, exogenous respiration, and photosynthesis.

2. Effects on growth have been studied by a method involving comparison of the growth curve of irradiated cells with growth curves for various known dilutions of untreated cells. Growth is inhibited exponentially with increasing dosage.

3. Except for a small transitory stimulation the endogenous respiration is not affected by dosages which severely inhibit other phases of metabolism.

4. Exogenous respiration of glucose or acetate is severely inhibited by UV. With increasing dosage the survival curve shows an exponential-decay toward the level of endogenous respiration. The effect of a given irradiation is a function of the product of intensity times time and is not otherwise dependent on the intensity used.

5. Following UV irradiation the rate of photosynthesis remains constant only for the first 30 to 50 minutes and then gradually falls off toward a final rate by a course which is interpreted as the utilization of some material in a first-order reaction.

6. Inhibition of photosynthesis, in terms of the initial rate, is described by a logarithmic survival curve which begins as a straight line but bends downward at higher dosages. Inhibition is not a function of dosage alone but is produced more effectively by irradiation at higher intensities.

7. Quantitative comparison has been made of the sensitivities of the 4 cellular processes to UV. The order of decreasing sensitivity is glucose respiration (1), growth (1/3.7), initial photosynthesis (1/25), and endogenous respiration (0). The final rate of photosynthesis shows a sensitivity approximating that of growth.

8. At least three different types of inhibition by UV are required to explain the observations. One of the effects is reasoned to be localized in a cellular component operating in the oxidative assimilation of exogenous substrate but not otherwise required for cellular syntheses. At least two other

types of effect are needed to explain the inhibition of photosynthesis but have not submitted to further identification by present techniques.

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THE RELATIONSHIP OF THE CELL SURFACE TO METABOLISM

VI. THE CHEMICAL NATURE OF URANIUM-COMPLEXING GROUPS OF THE CELL SURFACE[1]

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FOUR FIGURES

Uranium[2] in very low concentrations inhibits the metabolism of glucose by yeast cells (Booy, '40; and Barron, Muntz and Gasvoda, '48). Inasmuch as uranium influences metabolism by forming a complex with certain specific groups in the surface of the cell (Rothstein and Larrabee, '48; and Rothstein, Frenkel and Larrabee, '48), this element can serve as a useful tool in studying the relationship of these cell-surface groups to metabolism.

The metabolic function of the surface groups has not been unequivocally established. Barron et al. ('48) suggest that uranium complexes with proteins of the cell membrane, thereby reducing the permeability of the cells to sugars. On the other hand, Rothstein, Meier and Hurwitz ('51) believe that glucose is actively transported into the cell by a mechanism analagous to sugar resorption in the gut and kidney tubule, and that uranium complexes with groups which are an essential part of the transport mechanism. Unfortunately, the uranium-complexing groups of the cell surface cannot be identified with certainty on the basis of the metabolic studies. It was therefore felt necessary to undertake subsidiary chemical studies which might allow a more positive identification.

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[1] This paper is based on work performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, N. Y.

[2] Uranium in this paper refers to hexavalent uranium as typified by uranyl nitrate. This salt in aqueous solution is completely dissociated into uranyl ion (UO_2^{++}) and nitrate ion.

Some of the chemical properties of the cell-surface groups have been determined previously (Rothstein, Frenkel and Larrabee, '48). For example, the complex contains a 1 to 1 mol ratio of uranium to surface-groups, and it has a dissociation constant of about 3×10^{-7} at pH 3.5. In the present paper, the chemical properties of the uranium cell-surface complex have been explored in more detail and a comparison has been made with the properties of complexes formed between uranium and a large variety of known biochemical substances. In addition it has been shown that other cations will form complexes with the surface groups of yeast in competition with UO_2^{++}. On the basis of these studies, conclusions have been drawn concerning the chemical nature of the cell-surface groups and their role in metabolism.

METHODS

A number of techniques were used in the present work. Dissociation properties of uranium-complexes were determined by setting up a competition for uranyl ion between yeast cells and various complexing agents. The partition of uranium between yeast and supernatant in the presence of varying concentrations of each agent was determined by centrifuging the cells at high speed and analyzing for the uranium concentration of the supernate. The relative stabilities of the various complexes were calculated from mass-law equations. A different two-phase system, with an exchange resin as the solid phase, has been used previously by Schubert, Russell and Myers ('50) to determine stabilities of other complexes. A two-phase system offers certain definite advantages. A physical separation of the solid components of the system can be made without disturbing the equilibrium between free and complexed ions. Very low concentrations of uranium can be used because the technique is limited only by the sensitivity of the methods for determining the *total* uranium of the aqueous phase. Two of the methods for measuring uranium, the isotope technique (Rothstein, Frenkel and Larrabee, '49) and the photofluorometric technique (Neuman, Fleming, Carlson and Glover, '48), have adequate sensitivity.

Yeast suffers some disadvantages as a uranium complexor. First, it varies somewhat from sample to sample in its complexing capacity per milligram of yeast, introducing an error into the determinations. Second, it metabolizes some of the compounds whose uranium complexing capacity should be studied. For example, yeast cells contain phosphatases on the cell surface which split a large variety of phosphorylated compounds with the production of inorganic phosphate. However, the phosphatases can be inhibited completely by low concentrations of molybdate (Rothstein and Meier, '49). Fortunately, molybdate does not interfere with the formation of the uranium cell-surface complex or other uranium complexes. For this reason, in all experiments with phosphorylated compounds the yeast was poisoned with molybdate. Metabolism of other substances such as sugars was minimized by using a very short period of contact (3 minutes) during which little metabolism takes place, but which is long enough to allow uranium to reach its equilibrium distribution between cell surface and supernate.

The disadvantages of using yeast as a uranium complexor are more than offset by certain desirable qualities. Uranium very rapidly reaches an equilibrium distribution between the cells and supernate (Rothstein and Larrabee, '48). This distribution is constant for any given sample of yeast and it is not altered by several hours of starvation or by the pretreatment of the cells by a period of rapid metabolism with glucose as a substrate. Of primary importance, the use of yeast allows a *direct comparison* of the yeast-uranium complex with other complexes of uranium.

The experimental technique was rather simple. Fresh Baker's yeast (Standard Brands) was washed three times with 10 volumes of distilled water. In washing, the cells were separated at a low speed of centrifugation so that only the heavy cells were taken down, whereas light cells, cell fragments, dead cells and colloidal material to the extent of about 15% of the original yeast material were discarded. The heavy cells are the most viable cells and in later procedures can be completely separated from the supernate in two to three minutes by high-speed centrifugation (10,000 × gravity). The washed cells were starved for two hours with aeration. During the experimental procedures the yeast was kept under anaerobic conditions by bubbling a stream of nitrogen through the suspension with a sintered glass disc. This serves the dual purpose of reducing the metabolism to an extremely low level (Stier and Stannard, '36, have shown that endogenous metabolism is a strictly aerobic process), and it also provides adequate mixing of the yeast suspension.

Various concentrations of the substance under test were added to a suspension of yeast cells containing a fixed amount of uranyl nitrate. The final concentration of yeast cells was 10 mg wet weight per milliliter of suspension and that of uranium was 5×10^{-6} M (in a few experiments the final concentration of uranium was less than 5×10^{-6} M, but never below 4×10^{-6} M). Under these conditions a maximum of about 50% of the available yeast loci are complexed with uranium. After 5 minutes (except as noted above in the case of substances which could be metabolized by yeast in which the time interval was three minutes), the yeast was separated by high speed centrifugation (10,000 × gravity), and the supernates were analyzed for uranium by the isotope technique of Cohn and Hull as modified by Rothstein, Frenkel and Larrabee ('49). The alpha activity of the uranium was enhanced by the addition of U_{233} to the extent that 1% of the total uranium concentration used in the experiments (5×10^{-6} M) could be determined with an error of less than ± 5%.

In many cases it was desirable to know, for a particular complex, the mol ratio of uranium to complexing agent. Two techniques proved valuable — one involving the use of ferrocyanide and the other, of dialysis. In the case of the ferrocyanide technique, a competition was set up between the complexing agent and ferrocyanide for the available uranyl ion. The concentration of the uranium-ferrocyanide complex was determined colorimetrically from the orange color that developed, using a 500 mμ Farrand filter. If the complexing agent forms a more stable uranium complex than does the ferrocyanide, little color develops, unless sufficient uranium is added to saturate the complexing agent. Increasing amounts of uranyl nitrate were added to a solution containing potassium ferrocyanide (final concentration, 5×10^{-3} M) and a known concentration of complexing agent, all previously adjusted to pH 3.5. From the differences between the expected color (in the absence of complexing agent) and the observed color, the amount of uranium associated with the complexing agent could be calculated.

In the case of protein, the ferrocyanide technique could not be used because the protein precipitated with the ferrocyanide. The uranium binding capacity was therefore determined by a dialysis-equilibrium technique. Fifteen milliliters of a solution containing about 60 mg of protein was placed in a sausage casing (Visking). A known concentration of uranyl nitrate in 35 ml was placed in an Erlenmeyer and the casing was immersed therein. All solutions were previously adjusted to pH 3.5. The flask was gently agitated for 48 hours, which was ample time for the uranyl ion to equilibrate between the inside and outside compartments (checked experimentally with the uranium concentrations used). The outside fluid was then analyzed for uranium by the ferrocyanide technique. From the distribution of uranium between the inside and outside of the dialysis bag when varying concentrations were used, it was possible to calculate the uranium-binding capacity of the protein.

THEORY[3]

It has been shown previously (Rothstein, Frenkel and Larrabee, '48) that the formation of the 1:1 complex between yeast surface groups and uranium at pH 3.5 can be expressed in terms of the mass law, where

$$\mathrm{Ky} = \frac{(\mathrm{U})\ (\mathrm{Y})}{(\mathrm{UY})} \tag{1}$$

Similarly at a given and constant pH, the mass law expression for various uranium complexes can be expressed as

$$\mathrm{Kc} = \frac{(\mathrm{U})^{\mathrm{n}}\ (\mathrm{C})^{\mathrm{m}}}{(\mathrm{UnCm})} \tag{2}$$

In a system containing both yeast and complexing agent, there is a competition between yeast and agent for available uranium. If both sides of equation (1) are raised to the nth power and equation (2) divided by equation (1), then

$$\frac{\mathrm{Kc}}{\mathrm{Ky}^{\mathrm{n}}} = \frac{(\mathrm{UY})^{\mathrm{n}}}{(\mathrm{Y})^{\mathrm{n}}} \cdot \frac{(\mathrm{C})^{\mathrm{m}}}{(\mathrm{UnCm})} \tag{3}$$

Taking logs of both sides and transposing

$$\log \frac{\mathrm{Kc}}{\mathrm{Ky}^{\mathrm{n}}} = \mathrm{n} \log \frac{(\mathrm{UY})}{(\mathrm{Y})} + \mathrm{m} \log\ (\mathrm{C}) - \log\ (\mathrm{UnCm}) \tag{4}$$

If n is assumed to be 1, then

$$\log \frac{\mathrm{Kc}}{\mathrm{Ky}} = \mathrm{m} \log\ (\mathrm{C}) + \log \frac{(\mathrm{UY})}{(\mathrm{Y})\ (\mathrm{UCm})} \tag{5}$$

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[3] Symbols used here and in later parts of this paper are as follows:
UT, total uranium concentration
(U), free uranyl ion concentration
$\mathrm{Y_T}$, total concentraton of uranium-binding loci of yeast
(Y), concentration of free yeast loci
(UY), concentration of yeast-uranium agent
$\mathrm{C_T}$, total concentration of uranium-complexing agent
$\mathrm{C_{50}}$, total concentration of uranium-complexing agent necessary to give 50% of the uranium in the supernate under the conditions of these experiments.
(C), concentration of free complexing agent
(UnCm), concentration uranium-agent complex
Ky, dissociation constant for yeast-uranium complex
Kc, dissociation constant for agent-uranium complex
m, number of molecules of agent in the complex
n, number of molecules of U in the complex.

A plot of $\log {}_{(y)}\frac{(UY)}{(UCm)}$ against log (C) should be a straight line with a slope equal to — m and an intercept of $\log \frac{Kc}{Ky}$. If n is greater than 1, such a plot should not give a straight line. However, the data can then be replotted from equation (4) assuming n is equal to 2 or any other whole number. The assumed value of n which gives a straight line is the correct one. In addition to determining the value of m, from the slope, and the value for n by trial using different values, the relative stabilities of the various complexes, in terms of the dissociation constant Kc, can be calculated from the intercept — $\log \frac{Kc}{Ky}$. However, this method of determining the stabilities of complexes is awkward because of the extrapolation involved. A simpler method is available based on the experimental determination of the concentration of complexing agent associated with an equal distribution of uranium between the cells and the supernate. This parameter is called C_{50}.

The total uranium of the mixture (U_T) is made up of three fractions, the yeast-uranium complex (UY), the uranium-agent complex (UnCm), and the free uranyl ion (U), of which the latter two components constitute the uranium of the supernate. Under the conditions of the present experiments (10 mg of yeast and 5×10^{-6} M uranium), it was found that in the absence of competing complexing agents, about 95% of the uranium was taken up by the cells and only about 5% existed as free uranyl ion. If sufficient complexing agent (C_{50}) is added so that 50% of the uranium is to be found in the supernate, then according to equation (1), free uranyl ion (U) will constitute less than 2% of the total. The distribution of uranium will then be, (UY) = 50%, (U) = 2%, and (UnCm) = 48%. For purposes of this study, the 2% can be ignored and

$$\tfrac{1}{2} U_T = (UY) = n(UnCm) \tag{6}$$

Also,

$$C_{50} = (C) + m(UnCm) \text{ and } Y_T = (Y) + (UY) \tag{7}$$

Substituting equations (6) and (7) in equation (3)

$$\frac{Kc}{Ky^n} = \frac{(C_{50} - m/2n\, U_T)^m}{(Y_T - \tfrac{1}{2}U_T)^n} \cdot n(\tfrac{1}{2}U_T)^{n-1} \tag{8}$$

In cases where $n = 1$, the equation can be simplified to,

$$\frac{Kc}{Ky} = \frac{(C_{50} - m/2\ U_T)^m}{Y_T - \frac{1}{2}\ U_T} \tag{9}$$

In the experiments presented here, Y_T and U_T are kept constant. In nearly every case, $\frac{1}{2}\ U_T$ is very small relative to C_{50}, and can be dropped from the numerator, making $C_{50}{}^m$ directly proportional to Kc, the dissociation constant of the uranium complex.

It has been shown previously that the parameter Ky calculated from equation (1) is a constant, and it will be shown that Kc calculated from equation (9) is also a constant. However, these calculated constants are not the true dissociation constants of the uranium complexes, because of a number of simplifying assumptions which have been made. First, the participation of H^+ or OH^- ion in the complexes has been ignored, although it is known that the stability of each uranium complex studied is pH dependent. Second, no correction has been made for activities, which are unknown for many of the complexing ions, particularly the macromolecules. Third, in the case of macromolecules, it has been assumed that the concentration of active loci is to be used in the mass-law equation, rather than the actual molar concentration. Fortunately, under the conditions of the present experiments, the above assumptions do not invalidate a comparison of the stability of any given uranium complex with that of yeast. Thus, if the complexing agent under test is chemically similar to the yeast groups, the errors introduced by the simplifying assumptions will cancel out and Kc/Ky will be 1. If the complexing agent is not chemically similar to the yeast groups, errors may be introduced and Kc/Ky will not truly represent the ratio of the dissociation constants. The magnitude of the errors cannot be quantitatively evaluated. However, it seems probable that the ratio Kc/Ky is at least an approximate measure of the relative stabilities of the complexes at the pH of these experiments. It would be unlikely for a series of errors to combine in such a way as to produce a ratio of 1 for Kc/Ky if the complexing agent under test were not chemically similar to the yeast groups.

The parameters, Kc and Ky, have not been used directly to compare the stabilities of various complexes because such use might suggest that these constants are true dissociation constants. To avoid such connotations all comparisons have been made in terms of the C_{50} which carried no theoretical implications.

RESULTS

Method of expressing concentrations of complexing agents

In the case of yeast surface groups, and in the case of polymers of desoxyribonucleic acid[4] and of polyphosphate, molecular weights are unknown and it is necessary to express the concentrations in terms of the number of complexing loci. This assumes, of course, that all loci are equivalent, that each locus behaves independently, and that the probability of complex formation is a function of the concentration of loci, rather than of mols of each compound. In view of the fact that a large excess of complexing agent is used, only 1 uranyl ion, or, at most, a few uranyl ions are taken up by any one complexing molecule. Thus the influence of a complexed locus on the ability of adjacent loci to form complexes, such as was observed by Klotz ('50) for copper and proteins, is minimized, and the above assumptions should be valid.

In the case of yeast surface groups, the number of loci was determined from the maximal uptake of uranium. This technique is simple but can be applied only to a two-phase system. A suspension containing 10 mg of yeast, wet weight, per milliliter of suspension was found to contain about 1×10^{-5} mols of complexing loci per liter. For most of the other complexing agents, the ferrocyanide technique was used. Typical data obtained are given in figure 1, with pyrophosphate as a complexing agent. The sharp inflection point indicates that the pyrophosphate-uranium complex is considerably more stable than the ferrocyanide-uranium complex. The inflection point for 5×10^{-5} M pyrophosphate, obtained by extrapolation, was 9.5×10^{-5} M uranium. Thus each molecule of pyrophosphate took up about 2 molecules of uranium. Curves for most other compounds were similar in appearance, yielding the mol ratios of table 1. It is worth pointing out that with an increase in chain length in the phosphate polymers, there was an increase in the ratio of P to U in the complex from 1 to 1 for pyrophosphate, to 4 to 1 for high metaphosphate polymer. The failure of the ring structures, trimeta- and tetrametaphosphate to give a sharp inflection is due to the fact that these compounds do not form a very stable complex with uranium and they therefore do not compete successfully with ferrocyanide for uranyl ion.

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[4] One sample of desoxyribonucleic acid was supplied by Dr. Dounce, Department of Biochemistry, University of Rochester School of Medicine and Dentistry. This preparation is a pure, highly polymerized form of desoxyribonucleic acid. It contains no inorganic phosphorus, less than 2% labile phosphorus, and the theoretical amount (within 1%) of ester phosphate. A second sample was obtained commercially (Nutritional Biochemicals).

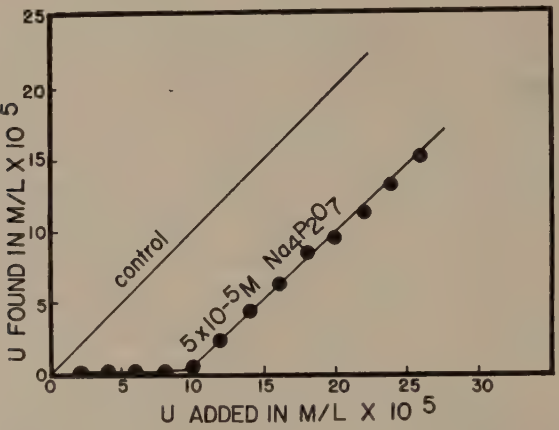

Fig. 1 Complexing of uranyl ion with inorganic pyrophosphate determined by ferrocyanide technique.

Ferrocyanide concentration was .05 M, pyrophosphate, 5×10^{-5} M, and pH 3.5.

In the case of the proteins, using the equilibrium dialysis technique, the data of table 2 were obtained. In the case of each protein a definite saturation point was reached, in contrast to the findings of Klotz ('50) concerning copper binding. With a serum albumin the saturation point was 1.5×10^{-5} mols of U per 60 mg of protein. One mol of uranium was therefore bound by 4000 gm of protein. Assuming a molecular weight of 70,000 for serum albumin (Cohn and Edsall, '43), each molecule of protein can bind 18 molecules of uranium. This is in close agreement with the value of 16 found for Cu binding (Klotz, '50). With egg albumin the maximal binding was 2×10^{-5} mols of uranium for 120 mg of protein. One mol of uranium was therefore bound by 6000 gm of protein. Assuming a molecular weight of 45,000 for egg albumin (Cohn and Edsall, '43), each molecule can bind 8 molecules of uranium.

TABLE 1

Mol ratios of uranium to phosphorus in various complexes as determined by ferrocyanide technique

Ferrocyanide concentration was .05 M and pH 3.5

COMPOUND	CONCENTRATION OF P IN M/1	CONCENTRATION OF U AT INFLECTION POINT IN M/1	MOL RATIO OF P TO U
Pyrophosphate	1×10^{-4}	$.95 \times 10^{-4}$	1/1
Tripolyphosphate	1.5×10^{-4}	1.0×10^{-4}	3/2
ATP [1]	1.0×10^{-4}	0.7×10^{-4}	3/2
Trimetaphosphate	no sharp inflection point		?
Tetrametaphosphate	no sharp inflection point		?
"Hexametaphosphate" [2]	6×10^{-4}	2×10^{-4}	3/1
High metaphosphate polymer [3]	1.3×10^{-4}	$.33 \times 10^{-4}$	4/1
Desoxyribonucleic acid	1.65×10^{-4}	$.43 \times 10^{-4}$	4/1

[1] The curves for ATP are difficult to interpret because they do not parallel the control values after the inflection point. Thus these data are open to question.

[2] "Hexametaphosphate" is a misnomer. According to Van Wazer ('50), "hexametaphosphate" is a mixture of linear polymers with an average chain length of 193 units. It is at least partially dialyzable.

[3] High metaphosphate polymer was prepared according to the method of Malmgren ('48) and has a molecular weight of about 1,000,000. It is non-dialyzable.

The binding of uranium by proteins is due primarily to free carboxyl groups (Dounce et al., '49). Egg albumin contains 46 free carboxyl groups, and serum albumin, 80 (Brand, '46). Thus in the case of each protein, the maximal number of uranyl ions that can be bound accounts for only about $\frac{1}{5}$ of the available free carboxyl groups. This suggests that, as in the case of copper, only those carboxyl groups which have adjacent accessory groups such as carboxyl and hydroxyl can form stable complexes with uranyl ion. Of course it must also be considered that at pH 3.5, which is below the iso-electric point, some of the free carboxyl groups are not dissociated and may for this reason fail to participate in complex formation.

TABLE 2

The binding of uranium by proteins as determined by equilibrium-dialysis technique

Fifteen milliliters of protein solution was placed inside of a dialysis sac (Visking sausage casing). This was allowed to equilibrate with 35 ml of uranyl nitrate solution placed outside of the sac. The equilibrium concentration of uranium in the outside compartment was determined by the ferrocyanide technique. The pH of each solution was 3.5

PROTEIN ADDED	CONC. OF UO_2^{++} ADDED TO OUTSIDE COMPARTMENT	EXPECTED EQUILIBRIUM CONC. OF UO_2^{++} IF NO BINDING OCCURS	ACTUAL CONC. OF UO_2^{++} FOUND	PROTEIN BOUND UO_2^{++} (EXPECTED — ACTUAL UO_2^{++} CONC.)
	mM/l	*mM/l*	*mM/l*	*mM/l*
0.4% serum	.40	.28	.10	.18
albumin	.50	.35	.11	.24
(Fraction IV)	1.0	.70	.46	.24
	1.5	1.05	.73	.32
	3.0	2.1	1.8	.3
	5.0	3.5	3.2	.3
0.8% egg	.40	.28	.01	.27
albumin	1.0	.70	.30	.40
(crystalline	1.5	1.05	.60	.45
	3.0	2.1	1.7	.4
	5.0	3.5	3.1	.4
	6.0	4.2	3.8	.4
Control	10	7.0	7.0	no protein added

Effect of complexing agents on uranium distribution

As mentioned previously, with a uranium concentration of 5×10^{-6} M and a yeast concentration of 10 mg/ml, only about 5% of the uranium remains in the supernate. On the addition of a complexing agent (provided of course that the complex is soluble), the amount of uranium in the supernate increased. Curves for a number of agents are shown in figure 2. These varied widely in their position on their concentration axis. For example, the concentration of "hexametaphosphate" necessary to give 50% of the uranium in the supernatant was 2.5×10^{-5} compared to 2.8×10^{-2} M for orthophosphate, a 1000-fold difference. Obviously the concentration of agent necessary to compete successfully with the cells for available uranium is an inverse function of the stability of the uranium-agent complex. Low concentrations of a strong complexor

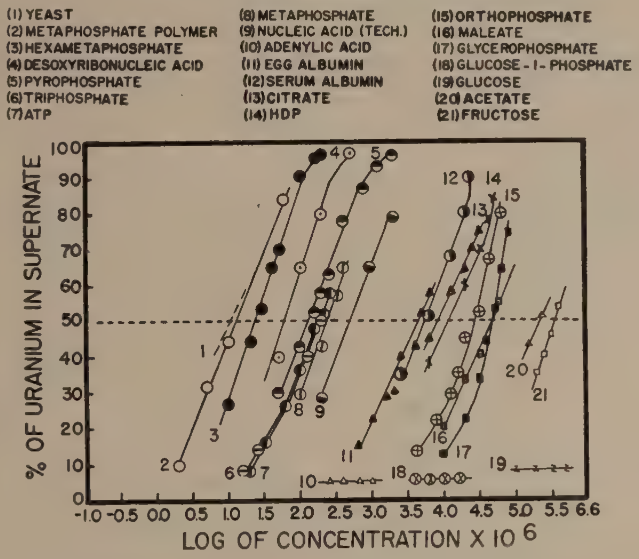

Fig. 2 Distribution of uranium between cells and supernate in the presence of various concentrations of complexing agents.

Yeast concentration was 10 mg wet weight per milliliter, U was 5×10^{-6} M and pH 3.5.

will be as effective as high concentrations of a weak complexor. The data of figure 2 represent essentially a spectrum of the stabilities of various uranium complexes at pH 3.5. The curves on the left represent the more stable complexes and those on the right, the less stable complexes.

The data of figure 2 were tested against the mass-law by the use of equation (5), which is a mass-law derivation. In figure 3, log (UY)/(Y)(UCm) was plotted against the log of (C) for some representative complexing agents. In each case a reasonably straight line was obtained. The same was true for all the other complexing agents for which adequate data were available to allow a curve to be drawn. This indicates first that the mass-law is applicable to the system, and second that the value of n, the number of uranium molecules entering into each complex molecule, is one. At first glance, it may seem strange that in every case, the value of n is 1, particularly when it can be shown that each molecule of some of these complexing agents are capable of binding more than 1 molecule of uranium. For example, pyrophosphate and triphosphate, in-

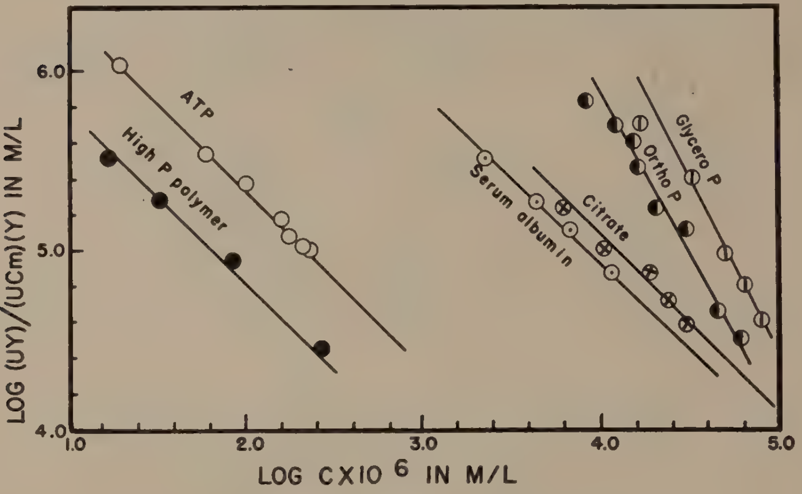

Fig. 3 Mass-law plot of some of the data of figure 2.

vestigated by the ferrocyanide technique, were found to have mol ratios of 2 to 1 in each case. With polymers, each molecule may bind many molecules of uranium. The apparent discrepancy between the mol ratio by ferrocyanide technique and the value of n of 1 by using equation (5) is due to the experimental technique. In the ferrocyanide technique *an excess of uranium is always used,* and the complex studied by this technique is that with the *highest* mol ratio of uranium to agent. In the data of figure 2, on the other hand, there is always *an excess of complexing agent.* The complex which is formed is that with the *lowest* mol ratio of uranium to agent.

The slope of the lines of figure 3 should, according to equation (5), be equal to m, the number of molecules of agent in the complex. In the case of high polymer, ATP, serum albumin and citrate,[5] the slopes were equal to 1. Other agents for which sufficient data were available also gave slopes of 1, with the exception of orthophosphate and glycerophosphate. These had slopes equal to 2, undoubtedly due to the fact that these substances at pH 3.5 are monovalent, whereas the others are polyvalent.

The straight lines found in figure 3, as well as the reasonable values of m and n obtained, lend credence to the hypothesis that the yeast-uranium complex obeys the mass-law, and that the technique used in these experiments is a valid one.

Relative stabilities of uranium complexes

The position of each curve of figure 2 on the concentration axis, which is a measure of stability of each complex, is most adequately measured by the parameter, C_{50}. In order to determine the stability relative to the yeast complex it is necessary to know the theoretical C_{50} for yeast itself. This can be readily calculated. It would be equivalent to the C_{50} for a soluble uranium complex having dissociation properties identical with the yeast-uranium complex. If such a complexing agent were present in a concentration exactly equal to that of the yeast loci, the uranium would be equally distributed between it and the yeast, meeting the conditions for the C_{50}. The C_{50} for yeast loci is therefore exactly equal to the concentration of yeast loci used in the experiments, 1×10^{-5} M. The mol ratio of uranium to yeast loci (n) is 1 (Rothstein, Frenkel and Larrabee, '48), so yeast in figure 2 (represented by a dotted line) is parallel to the curves for the other substances with an n of 1. The C_{50} for each complexing agent as well as the ratio, C_{50} for agent/C_{50} for yeast, are given in table 3.

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[5] The data for citrate was replotted assuming that the complex was a dimer (Neuman, Havill and Feldman, '49) and a fairly good straight line was obtained with a slope of 2. However, when the data were plotted, assuming that n was equal to 2, then a definite curve was obtained. Thus the method will differentiate a monomer from a dimer.

There was a wide spectrum of stabilities among the different complexing agents. The C_{50}'s varied from 3.8×10^{-1} for fructose to 1×10^{-5} for yeast loci, a 38,000-fold range. The weakest U-complexors were polyhydroxyl compounds such as fructose and mannose.[6]

Among the organic acids, the monocarboxylic acids such as acetate formed relatively unstable complexes, whereas the dicarboxylic acids such as maleic formed more stable complexes presumably due to the chelation. Citrate, a tricarboxylic acid, formed an even more stable complex because of multiple ring closure (Neuman et al., '49). The most stable complex involving carboxyl groups was that with proteins. Here undoubtedly accessory groups play an important role, including, possibly, the single phosphate molecule found in each protein molecule. As pointed out, the C_{50} was calculated on the basis that all of the loci form complexes of equal stability. This may not be true in proteins in which case the uranium would be unequally distributed among the loci. In view of the fact that insufficient uranium is present to satu-

[6] Mannose complexing was determined by polarographic technique (Dounce et al., '49). However, glucose and galactose did not complex. The 2 and 3 hydroxyl groups of the sugars must apparently be in the cis position in order to get complexing. Other polyhydroxyl compounds such as resorcinol will form complexes with U (Ware, '45).

rate any single locus of the protein, then any one locus that formed a much more stable complex than the others, would bind all of the uranium. The C_{50} based on a single locus would be 3.5×10^{-4} M for serum albumin and 5.6×10^{-4} M for egg albumin, and the relative stabilities, compared to yeast, 35 and 56.

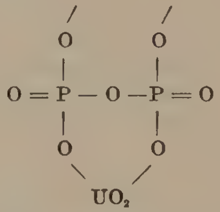
TABLE 3

A comparison of the stabilities of various uranium complexes at pH 3.5

COMPLEXING AGENT	C_{50}	RATIO OF C_{50} FOR AGENT TO C_{50} FOR YEAST
	M/l	
Yeast surface groups	1.0×10^{-5}	..
High polymer metaphosphate	1.3×10^{-5}	1.3
"Hexametaphosphate"	2.5×10^{-5}	2.5
Desoxyribonucleic acid	6.3×10^{-5}	6.3
Pyrophosphate	1.3×10^{-4}	13
Tripolyphosphate	1.9×10^{-4}	19
ATP	2.0×10^{-4}	20
Metaphosphate	2.5×10^{-4}	25
Desoxyribonucleic acid (commercial)	5.0×10^{-4}	50
Egg albumin	4.5×10^{-3}	450
Serum albumin	5.6×10^{-3}	560
Citrate	8.5×10^{-3}	850
Adenylic acid	$> 1.0 \times 10^{-2}$	> 1000
Trimetaphosphate	about 1×10^{-2}	about 1000
Tetrametaphosphate	about 1×10^{-2}	about 1000
Hexose diphosphate	1.3×10^{-2}	1300
Orthophosphate	2.8×10^{-2}	2800
Glucose-1-phosphate	$> 3.2 \times 10^{-2}$	> 3200
Maleate	5.0×10^{-2}	5000
Glycerophosphate	5.0×10^{-2}	5000
Acetate	2.2×10^{-1}	22,000
Fructose	3.8×10^{-1}	38,000
Glucose	> 1.0	$> 100,000$

The other carboxyl compound which forms a complex of physiological importance in animals is bicarbonate. This complex does not exist to any extent at the pH used in these experiments. Amino acids (except probably the dicarboxylics) and sulfhydryl compounds (cysteine and glutathione) do not form complexes with uranyl ion (Dounce et al., '49).

The last group of complexes to be considered are the phosphates. These vary considerably in stability. Compounds with a single phosphate group such as glycerophosphate, adenylic acid, glucose phosphate and inorganic orthophosphate form relatively unstable complexes. Hexose-diphosphate forms a more stable complex, about the same as citrate. The most stable complexes of uranium are those with compounds having a number of phosphorus atoms, such as nucleic acids, ATP, and inorganic linear polymers of phosphate. The stability of these complexes is related to their chelating ability, with ring closure which can be characterized as follows for pyrophosphate:



The two known ring structures trimetaphosphate and tetrametaphosphate form relatively weak complexes. In the case of the linear polymers, there is a marked increase in stability with an increase in molecular weight. Thus the high molecular weight polymer forms the most stable complex followed by "hexametaphosphate" and then by the pyro- and tripolyphosphate. The increased stability of the complexes with the higher molecular weight polymers is undoubtedly associated with the higher ratio of phosphorus to uranium in these complexes (see preceding section).

Of all the complexes studied, the most stable is the yeast complex. The only complexes which approach that of yeast in regard to stability are those of the polyphosphate compounds, particularly the high molecular weight polymer with a ratio relative to yeast of 1.3 followed by "hexametaphosphate" with a ratio of 2.5, and polymerized desoxyribonucleic acid with 6.3. All other substances tested formed complexes which were much less stable. On this basis, it is suggested that the yeast surface group is chemically similar to the high molecular weight polyphosphates.

Effect of pH on stability

It has been shown that the yeast-uranium complex is similar in its dissociation properties to the polyphosphate-uranium complex. In order to compare these complexes further, a study was made of the effect of pH on stability. "Hexametaphosphate" was chosen as a representative phosphate polymer, and citrate, as a representative of the carboxyl containing, effective, complexing agents. The organic acid complexes are known to increase in stability as the pH is raised (Feldman and Neuman, '51). No data are available for the yeast complex or the polyphosphate complexes.

The experimental technique was simple, involving a direct comparison of the effect of pH on the distribution of uranium between cells and complexing agent. A fixed amount of uranium and yeast was mixed with a fixed amount of citrate or polyphosphate, following the adjustment of each solution to the required pH. After 5 minutes the cells were centrifuged down and the supernatant analyzed for uranium. There were no appreciable shifts in pH during this procedure. With no added complexing agent (control), an increase in pH from 2.5 to 4.5,[7] resulted in a marked decrease in the uranium concentration of the medium (fig. 4), indicating an increased stability of the yeast-uranium complex. With citrate in the medium, there was also a marked redistribution of uranium as the pH was raised, which consisted of an increased uranium-binding by the cells. Thus the stability of the yeast-uranium complex was increased to a greater extent than was that of the citrate-uranium complex. In the case of the "hexametaphosphate,'' however, there was very little redistribution of uranium with a change in pH. In other words, the increased stability of the yeast-uranium complex at higher pH's *was matched by an equal increase* in the stability of the polyphosphate-uranium complex. Thus the polyphosphates are chemically similar to yeast surface loci not only because the uranium complexes possess stabilities of the same order, but also because they are influenced by pH in the same manner.

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[7] This pH range is chosen for two reasons. Above pH 4.5, the uranyl ion forms a hydroxyl complex, which might affect its distribution. Below pH 2.5, yeast, although not irreversibly damaged, begins to show some loss of ability to metabolize glucose. The total uranium-binding by yeast is unaffected by the shift in pH.

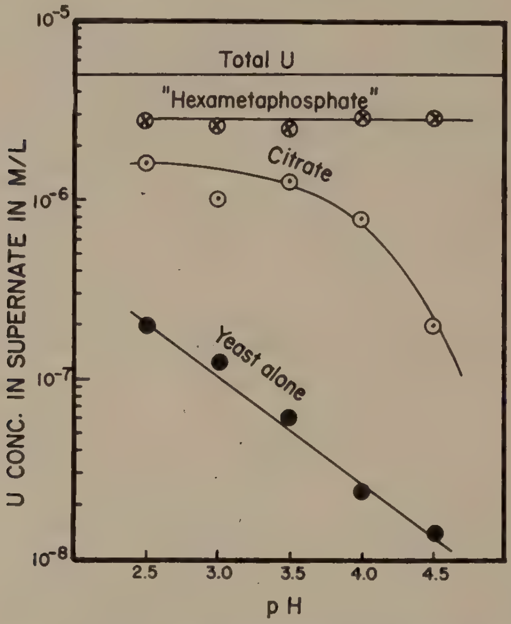

Fig. 4 The effect of pH on the distribution of uranium between cells and supernatant in the presence of citrate and ''hexametaphosphate.''
Yeast concentration was 10 mg wet weight per milliliter, uranium, 5×10^{-6} M, citrate, 1×10^{-2} M and ''hexametaphosphate,'' 2×10^{-4} M in terms of P.

Competition between uranyl ion and other cations for cell-surface loci

The previous sections of this paper have been concerned with a competition between complexing agents and yeast cell loci for available uranyl ion. It is also possible to set up a competition between uranyl ion and other cations for available yeast loci. The technique is the same as that already described. Various concentrations of competing cations are added to a system containing yeast and uranium. If the cation competes with uranyl ion, it will tend to displace it from the cell surface and thereby will increase the uranium concentration of the supernate. Data are given in table 4.

TABLE 4

The competition of bivalent cations with uranyl ion for cell surface loci as measured by uranium distribution between cells and supernate

The yeast concentration was 10 mg/ml; pH 3.5; total uranium concentration was 5×10^{-6} M. All cations were added as nitrate

CATION	TOTAL CONCENTRATION OF TEST CATION	% OF U IN SUPERNATE
	M/l	
Controls	0.0	5 [1]
Ba^{++}	1×10^{-3}	26
Mn^{++}	1×10^{-4}	6
Zn^{++}	1×10^{-3}	4
	1×10^{-2}	25
Be^{++}	1×10^{-3}	18
Mg^{++}	5×10^{-4}	4
	1×10^{-3}	7
	5×10^{-3}	9
	1×10^{-2}	16
Ca^{++}	1×10^{-2}	23
	3×10^{-2}	28
	5×10^{-2}	34
	7×10^{-2}	36
	30×10^{-2}	93
Na^{+}	1×10^{-1}	8
K^{+}	1×10^{-1}	9

[1] Mean of 6 determinations ranging from 2.5 to 8.4

Of the cations tested, Ba^{++} and Zn^{++} were the best competitors. In the presence of a concentration of 1×10^{-3} M, Ba^{++} or Zn^{++}, about 25% of the uranium was found in the supernate. In the absence of competing cations, only about 5% of the uranium was found in the supernate. Thus, this concentration of competing cations has displaced about 20% of the uranium from the cells. The concentration of uranium was only 5×10^{-6} M or 1/500 as high as that for the competing cation. Thus the complex of uranium with cell-surface loci is considerably more stable than that of Ba^{++} and Zn^{++}.

Next to Ba^{++}, Be^{++} was the best competitor with 1×10^{-3} M concentration displacing 13% of the uranium. Ca^{++} and Mg^{++} formed less stable complexes with 10^{-2} M necessary to displace an appreciable quantity of uranium. Mn^{++} at 1×10^{-3} M did not compete. Attempts to study the effects of Cu^{++} and Hg^{++} were unsuccessful due to the fact that these ions, in the necessary concentrations, seriously interfere with plating procedure for uranium. The monovalent cations, Na^{+} and K^{+} even at 0.1 M concentrations did not show any appreciable displacement of uranium.

DISCUSSION AND CONCLUSIONS

It is suggested on the basis of the work reported here that the uranium complexing loci of the yeast cell are chemically similar to the polyphosphates. If this conclusion is correct, then two further conclusions follow. First, the polyphosphate must exist on the surface of the yeast cell because uranium acts on the cell surface (Rothstein and Larrabee, '48). Second, the polyphosphate must be intimately involved in hexose metabolism, because uranium specifically inhibits the metabolism of the hexoses (Rothstein, Meier and Hurwitz, '51).

In recent years there has been considerable interest in metaphosphate (polyphosphate) from a biological point of view. These compounds have been isolated from yeast (Wiame, '49; Schmidt and Hecht, '49; and Juni et al., '48), bacteria (Ebel, '49) and fungi (Ingelman and Malmgren, '50). In the latter case, the metaphosphates have been shown to be highly polymerized. A number of studies have been made on the metabolic role of the yeast metaphosphates. Although no specific role can be assigned to these compounds at the present time, there is no question that they are intimately concerned with the metabolism of phosphate and of sugars. For example, during starvation the metaphosphate content of yeast is gradually depleted, whereas the addition of glucose plus inorganic phosphate results in an immediate spectacular increase (Wiame, '49). Up to 20% of the phosphate of the cell may be found as metaphosphate. If radioactive phosphorus is used in such experiments, it is found that a metaphosphate fraction shows a very high turnover (higher than any compound except the nucleic acids) of phosphorus, after glucose is added (Juni et al., '48). There is a suggestion that phosphate uptake by cells, which is considered to be an "active" energy requiring process (Kamen and Spiegelman, '48; and Sachs, '48) involves the formation of metaphosphates (Schmidt and Hecht, '49).

The metaphosphate polymers consist of chains of phosphorus atoms linked to each other through oxygen atoms. Such linkages are relatively unstable (labile) and are readily hydrolyzed by mild acid (7 minutes in 1 N HCl at 100°C.). Each such linkage contains a considerable potential of energy, of the same order of magnitude as the phosphorus linkages in ATP. The formation of metaphosphates must therefore involve the expenditure of considerable metabolic energy by the cell. Conversely the breakdown of the metaphosphates must also liberate considerable quantities of energy which might be available for synthetic reactions. The metaphosphate polymers probably serve as a storehouse of high energy phosphate linkages which can be made available for phosphorylation reactions. The energy for synthesis of metaphosphate is undoubtedly supplied by sugar metabolism. The source of phosphate can be the orthophosphate of the medium, thus providing a mechanism for phosphate uptake.

Normally the phosphorylation reactions require the presence of bivalent cations such as Mg^{++}, Mn^{++} and Ca^{++}. Presumably the cell-surface polyphosphate compounds are present in the form of a complex with these ions. Uranyl ion would compete with the Ca^{++} or Mg^{++} for available polyphosphate loci, forming a highly undissociated complex which is physiologically inactive. Such a hypothesis is in accord with the metabolic effects of uranium. In a previous paper (Rothstein, Meier and Hurwitz, '51), based on metabolic studies, it was suggested that uranium inhibits phosphorylation reactions, occurring on the surface of the cell. These reactions may be involved in the active transport of glucose into the cell.

SUMMARY

1. Uranyl ion forms complexes with a large variety of substances of biological interest. The active complexing groups may be hydroxyl, carboxyl or phosphate.

2. The number of complexing loci in certain macromolecules has been determined at pH 3.5. For example, serum albumin has 18 loci, egg albumin, 8. Among the polyphosphates, the mol ratio of phosphate to uranium in "hexametaphosphate" is 3 to 1, in highly polymerized metaphosphate and in desoxyribonucleic acid, 4 to 1.

3. In the presence of an excess of complexing agent, the data can be fitted by equations derived from the mass-law.

4. Yeast forms the most stable complex with uranium. The only substances which form complexes of about equal stability are the high molecular weight polymers of phosphate. Other substances formed much less stable complexes.

5. pH has a marked effect on the stability of the uranium complexes. The polyphosphate complexes are affected by pH in the same way as is the yeast complex, whereas carboxyl-complexes, as represented by citrate, are affected in a markedly different manner.

6. Other bivalent cations such as Ba^{++}, Ca^{++}, Be^{++}, Mg^{++} and Zn^{++} can compete with uranyl ion for the cell-surface loci. Na^+ and K^+ do not.

7. The data presented here are consistent with the hypothesis that the uranium-complexing loci of the cell surface are polymers of phosphate and that uranyl ion inhibits sugar metabolism by forming relatively undissociated complexes with these polyphosphates (possibly replacing Ca^{++} or Mg^{++}).

The polyphosphates are presumably necessary for phosphorylation reactions involved in the transport of glucose into the cell.

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EFFECTS OF POTASSIUM AND RUBIDIUM ON THE RESTING POTENTIAL OF MUSCLE [1, 2]

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THREE FIGURES

There is considerable support, although at best always with certain reservations, for the view that the Bernstein potassium diffusion potential theory provides a fairly adequate explanation of the resting membrane potential of cells. In principal confirmation of this theory is the general verification of its prediction that there is a linear relation between the logarithm of the external potassium concentration and the potential. This has been found to hold for a variety of cells (e.g., Fischer, '24; Hegnauer et al., '34; Cowan, '34; Steinbach, '40a, b; Curtis and Cole, '42; Shanes, '44); and yet it should be noted that, as found in some of this work, the linear relation fails to hold when the external experimental potassium concentrations are quite low.

A recent source of doubt of the Bernstein theory has been expressed by Lorente de Nó ('47), who found, contrary to the expectation of the supposed diffusion mechanism, that the potassium depolarization of the frog sciatic nerve trunk is an extremely slow process, taking many hours for completion. He therefore claimed that, where found, the linear relation between log K and the potential is a mere coincidence. Quite apart from the pertinence of Lorente de Nó's basic observation to the question of the validity of the Bernstein theory,

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[2] The material of this paper comprises an abstract of part of the Master's Thesis of Mr. Mandel.

the experimental meaning of this observation has been seriously questioned by Feng and Liu ('49a, b) who claim that the slowness of the course of K-depolarization as observed by Lorente de Nó is due to the diffusion barrier set up by the nerve sheath. In experimentally prepared sheathless frog nerve, these workers found that the maximum depolarization due to a given concentration of potassium was established very quickly, in a time of the order of some minutes, and that the magnitude of the equilibrium potential, except for low concentrations, was a linear function of the log K. In other publications (Feng and Liu, '50; Lorente de Nó, '50) the controversy concerning the significance of the nerve sheath as a diffusion barrier is continued, and despite seemingly cogent demonstrations on both sides of the argument, contradictions still exist which are in need of further resolution.

Another apparent objection to the Bernstein theory is found in the work, e.g., of Gallego and Lorente de Nó ('47) which showed that the depolarization of nerve (with sheath) produced by 0.1 M RbCl is almost identical with that due to 0.1 M KCl. It is asserted that this depolarization cannot be explained by the Bernstein theory. And, Feng and Liu ('49b) take somewhat the same view of their results with rubidium, which were obtained on sheathless nerve, and which showed similar effects to those caused by potassium, including the linear relation, although at equal concentrations the Rb-depolarization was always less than that due to potassium. On the basis of their findings, the Chinese workers state that the linear relation for Rb ''cannot be understood by any reference to the concentration ratio of rubidium inside and outside.'' They are thus led to question the Bernstein theory and to postulate, as an alternative explanation of the action of these cations, that they enter interchangeably into a chemical reaction with some constituent of the tissue and thereby produce their respective depolarizations.

The present research has been carried out in order to compare the kinetics of the depolarization by potassium, reported by Feng and Liu ('49b) for sheathless nerves, with similar curves for muscle. Further studies of the effects of the rubidium ion on the membrane potential will also be described and the relation of the results to the potassium diffusion theory will be discussed.

METHOD

The sartorius muscle of the frog (Rana pipiens) was used in the experimental work. The muscle was carefully excised and equilibrated in oxygenated, phosphate buffered (pH 7.2) Ringer's solution for one hour. The apparatus consisted of a voltmeter (Central Scientific — Catalogue no. 20965), a pair of calomel electrodes, and a chamber of paraffin and polystyrene. The voltmeter, although designed as a titration and pH meter, was adaptable for the measurement of resting potentials since it has a relatively high input resistance (5 megohms). The scale is calibrated into two millivolt divisions and the voltage could be estimated to 0.2 of a millivolt. The chamber was prepared so that one portion of the muscle was dipping into an experimental solution while another was dipping into Ringer's solution. All experiments were performed at room temperature which averaged about 20°C.

It was realized that a measurement of the potential in this manner (the indirect method) does not constitute a quantitative determination of the true membrane potential as found by the direct method (Curtis and Cole, '42; Graham and Gerard, '46; and Ling and Gerard, '49). However, it may be assumed that the effects of ions will be proportionately the same by either method; that is, if the depolarization by an agent such as isotonic KCl is adopted as complete or 100%, then the effects of solutions containing some other ion or a smaller concentration of potassium ions may be determined as a per cent depolarization by either the direct or the indirect method. For this reason and to overcome difficulties presented by individual variations among muscles each determination was begun with Ringer's solution in both sections of the chamber and, at some time during the experiment, a solution of 0.1 M KCl was placed in the "experimental" section. The difference between the resulting potential values was used to represent 100% depolarization of the membrane, and any other depolarization change caused by the various agents tested was compared to this on a percentage basis.

A number of experimental solutions was used. In studying the effects of potassium, this ion was present, in Ringer's solution, in the following concentrations expressed in mg %: 7.8 (normal Ringer's), 18.0, 23.0, 28.1, 38.3, 48.4, 68.7, and 527 (0.1 M KCl). The effect of the rubidium ion was studied with a solution of 0.1 M RbCl. In the potassium experiments, readings were taken and recorded every minute or two for the first 5 or 10 minutes, but once the voltage approached the equilibrium level the interval between readings was extended to 5 or 10 minutes. In the rubidium experiments, a similar procedure was used, but it was necessary to take readings over much longer periods of time.

RESULTS

Potassium

A typical stepwise depolarization curve produced by a series of experimental solutions containing an increasing amount of potassium is shown in figure 1. The zero potential was measured with Ringer's solution, which was then replaced, in turn, by each of the experimental solutions. Complete depolarization was measured by replacing the last experimental solution (68.7 mg % K) with 0.1 M KCl and, finally, Ringer's solution was used to test for reversibility. It may be seen that there is a progressively greater degree of depolarization with each increase in the concentration of potassium above that of Ringer's solution and that this depolarization is completely reversible. Furthermore, each solution produced a relatively large, rapid potential change at first and the rate of change then gradually decreased until the curve reached an equilibrium level where the potential became constant. The time required for each step in the depolarization to attain a constant level ranged from 15 to 40 minutes.

The inset in figure 1 shows that a linear relationship is displayed when the maximum value of the potential (as per cent depolarization) is plotted against the logarithm of the concentration of potassium in the experimental solution, except in the region of lowest concentrations. The potential values used to prepare this graph were the averages obtained from 11 muscles.

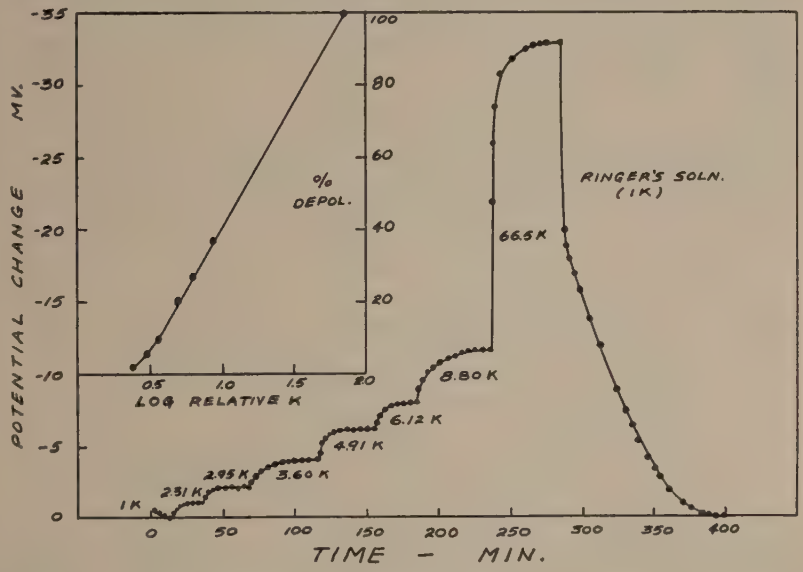

Fig. 1 A typical stepwise depolarization curve for a series of solutions of increasing K concentration. Inset: the percentage depolarization plotted as a function of the log K, with K expressed as a multiple of its concentration in normal Ringer's solution (averages of 11 muscles).

In many of the experiments presented in this paper, it was necessary for the muscle to undergo treatment with various solutions as well as equilibration in oxygenated, buffered

Ringer's solution for periods of several hours. At the end of many of these experiments, complete depolarization by 0.1 M KCl was followed as a control. In order to insure the validity of this procedure, the effect of prolonged equilibration and previous treatment with KCl on the quantitative and kinetic aspects of depolarization by 0.1 M KCl was determined. This determination, as illustrated by figures 2 A and B, also serves as a further study of the kinetics of the depolarization by 0.1 M KCl and demonstrates the repeatability of such depolarizations.

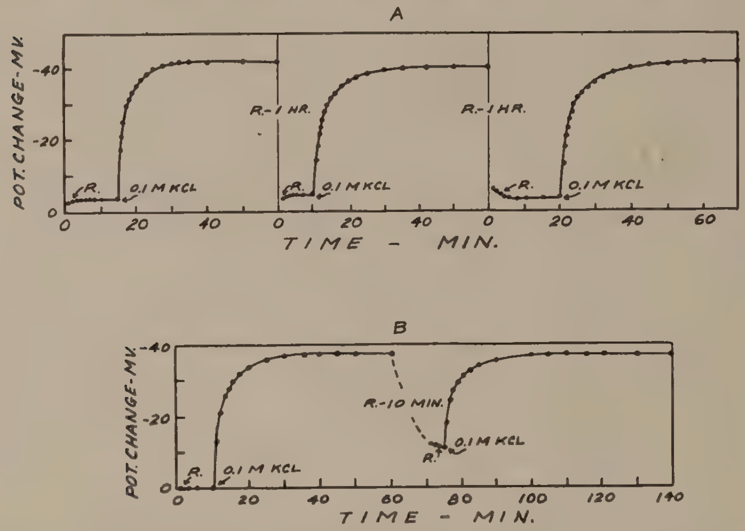

Fig. 2 Two series of successive depolarizations by 0.1 M KCl showing the kinetics, reversibility, and repeatability of the process. The equilibration between successive runs refers to reversal of the depolarization in normal Ringer's (R). A. Three successive runs separated by intervening reversals of one hour each. B. Two runs with intervening reversal for 10 minutes.

In figure 2 A, a series of three depolarizations by 0.1 M KCl are presented with the interjection of one and one-half hours of equilibration in oxygenated, buffered Ringer's solution after each of the first and second depolarizations. There is no significant effect of equilibration of previous KCl treatment on the maximum value obtained in the three cases. The average maximum value is — 41.4 ± 0.05 mv. The reversibility is complete; that is, there is no significant change in the potential produced by Ringer's solution after equilibration for one and one-half hours. The average value for this potential is — 3.9 ± 0.2 mv.; therefore, the average net change brought about by 0.1 M KCl is — 37.5 ± 0.7 mv.

While there is no significant change in the initial and final values of the potential in successive depolarizations by KCl, there is a slight progressive change in the kinetics of the depolarizations. With minor variations, each depolarization curve appears to lag about 5 minutes behind the preceding one. The first, second, and third curves reach their maxima after treatment with 0.1 M KCl for 20, 25, and 30 minutes respectively. The rapidity of the depolarization is indicated by the fact that the membrane is more than 80% depolarized after 5 minutes and almost 95% depolarized after 9.5 minutes of KCl treatment, according to the first depolarization curve in figure 2. The corresponding values are somewhat smaller for the other two depolarization curves.

In order to show that the maximum potential produced by 0.1 M KCl is not dependent upon complete reversal via prolonged equilibration, experiments were done involving incomplete reversal. These are illustrated in figure 2 B. Incomplete reversal following KCl depolarization was effected by a 10 minute period of equilibration in Ringer's. Subsequent depolarization by 0.1 M KCl remained essentially unchanged.

Rubidium

The effect of the rubidium ion on the membrane potential was studied by means of a series of experiments in which 14 muscles were used. The average change in potential produced by 0.1 M RbCl was found to be — 22.8 ± 3.0 mv. and the values ranged from — 16.6 to — 31.6 mv. The effect of 0.1 M KCl on each muscle (or, in some cases, each pair of muscles from the same frog) was determined first as a control. From these values, the per cent depolarization produced by 0.1 M RbCl was calculated and the average was found to be 61.1 ± 4.1%.

This partial membrane depolarization produced by rubidium has been reported by many investigators (Höber, '05 and '45; Seo, '24; Sugi, '34; and Höber et al., '39 for frog muscle — Cowan, '37 and Guttman, '40 for crab nerve — Netter, '27; Höber, '46; and Feng and Liu, '49b for frog nerve — and others). Seo ('24), using one part salt (isotonic) and one part sucrose, observed that KCl produced a potential of − 38.9 mv., while that produced by RbCl was − 29.1 mv. (or 74.7% depolarization). Sugi ('34) found that 0.12 N KCl produced a potential of − 31 mv., while 0.12 N RbCl yielded − 23 mv. (or 67.6% depolarization). The range of values determined for the effect of 0.1 M RbCl in the present investigation was − 16.6 to − 31.6 mv. (or 55.8 to 75.4% depolarziation), which is extensive enough to include the values obtained from the data of Seo and Sugi.

As shown in figure 3 A, the rubidium ion has another effect in addition to partial membrane depolarization: there is a temporary depression of KCl depolarization after treatment with rubidium. The average potential (5 muscles) produced by 0.1 M KCl following depolarization by 0.1 M RbCl and one hour of equilibration in Ringer's solution is − 14.5 ± 1.0 mv. or 34.8 ± 2.4% of the normal 0.1 M KCl depolarization. However, if the muscle is allowed to equilibrate for two hours instead of one, the average potential (three muscles) is − 24.6 ± 2.0 mv. or 76.3 ± 1.4% depolarization. Thus, the membrane potential gradually tends to return to its normal value.

Figure 3 B shows that the recovery of the normal KCl depolarization curve after RbCl treatment is more rapid if the muscle is kept in the RbCl solution for only one hour; that is, if the RbCl depolarization is not allowed to reach its maximum value. Thus, the duration of the depression of KCl depolarization is a function of the duration of the RbCl treatment as well as the duration of equilibration in Ringer's solution following the RbCl treatment.

In addition to the above relationships, the difference between the kinetics of the depolarization curves produced by 0.1 M KCl and 0.1 M RbCl, are illustrated by the curves in figure 3. RbCl depolarizes much more slowly than KCl. The former takes, on the average, about two hours to reach a maximum and about 60 minutes to reach 90% of this maximum. The latter, on the other hand, reaches a maximum value in less than 30 minutes and reaches 90% of this maximum in 5 to 10 minutes. The shift of the potential from − 4.0 mv. before RbCl treatment to + 4.0 mv. after RbCl treatment is not a constant feature of the rubidium experiments and, therefore, should not be considered dependent upon the presence of the rubidium ion.

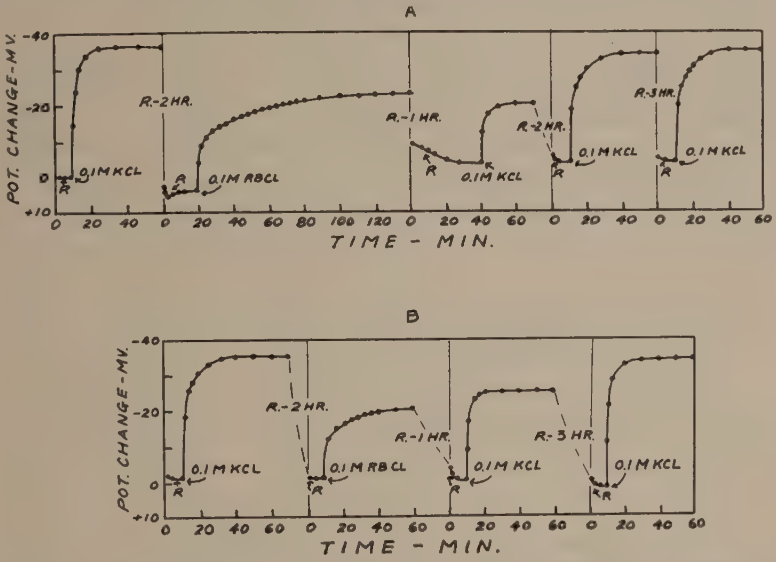

Fig. 3 Two series of depolarizations by 0.1 M RbCl and 0.1 M KCl illustrating the differences in kinetics and magnitudes of the potentials by each salt, and the effect of Rb on subsequent K-depolarization. R = Ringer's solution. Time of action of Rb: A, 2 hours; B, 50 minutes.

DISCUSSION

Our results are of theoretical interest in relation to two currently significant problems regarding the nature of resting cell potentials. The first is the role of diffusion in the kinetics of depolarization by agents such as potassium and rubidium; and the second is concerned with the meaning of the linear relation between the potential and the logarithm of the external concentration of either potassium or rubidium.

We have demonstrated that the depolarization of the frog sartorius muscle by potassium is a quite rapid process. On the average, 0.1 M KCl, at 20°C., causes full development of its depolarization in about 20 to 30 minutes, and 80% of this is effected in 4 minutes. Similar results have been obtained by Shanes ('48). Thus, these results for the frog sartorius are kinetically like those of Feng and Liu ('49a, '50) on sheathless nerve, for an examination of their curves, which were obtained at about 10°C., shows that the full effect of isotonic KCl was established in 30 to 40 minutes and 80% depolarization was developed in two to three minutes. Now, Lorente de Nó ('47, '50) believes that the quickness of the action of potassium on sheathless nerve, which is so different from the extreme slowness of depolarization observed by him ('47) on intact nerve, is due to the alteration of the actual depolarizable structures — the nerve fibers — by the work involved in the removal of the sheath. Our results would seem to invalidate this view; for, though they were obtained on muscle instead of nerve, the muscles suffered no alteration comparable to unsheathing a nerve; thus, their fibers must have been quite intact, and yet depolarization was very rapid. It is evident that depolarization by potassium can be very rapid in muscle fibers that could hardly be said to be damaged.

It is of interest to study the kinetics of the depolarization by 0.1 M KCl more quantitatively. It is not easily possible to do this unequivocally for the entire course of a depolarization curve. When diffusion of the KCl into the muscle has progressed to a certain moment, it is clear that the salt will be present at a diminishing concentration the closer the point for consideration is to the center of the muscle thickness. At each point we may assume that the potential of the corresponding fiber is a function of the log K at that point. The difficulty then arises of relating the potential measured at the muscle surface to these varying potentials throughout its thickness. A rough, equivalent circuit analysis proves that this is a hopelessly complicated task. But this analysis indicates that when diffusion is far advanced and the KCl is thus rather uniformly concentrated within the muscle, that, then, the surface potential measurement may be assumed to represent a fair approximation of the average of the potentials of the separate fibers.

Hence, we will consider certain features of the diffusion of KCl into the muscle up to the time (9.5 min.) at which we measure that the muscle is 95% depolarized. We now assume that, at this time, the average degree of saturation of the muscle interstitial space, in respect to the external 0.1 M KCl, is given by the equilibrium conditions represented in the linear relation curve of figure 1. Thus, at 95% depolarization, the K concentration is 59 × that of normal Ringer's solution; and since the external 0.1 M solution of KCl is equivalent to 66.5 × the Ringer's concentration, the degree of saturation for the 95% depolarization level is $59/66.5 = 0.90$.

We may now use this number to calculate the diffusion constant of KCl in the muscle, using the theory of diffusion processes as developed by Hill ('28). For this purpose, assume that our system corresponds to the case of two-sided diffusion of a substance into an infinite plane sheet. The muscle, of course, is not quite such a sheet, especially since the diffusion of KCl into it, during the very short time involved, can be only by means of the extracellular space. Thus, the diffusion will occur along irregular paths, and not in straight lines — a point we will return to later. Moreover, the limitation of diffusion through only the interstitial space means that, in a given time a much smaller *amount* of KCl can pass into the muscle than if it were free to move into the intracellular as well as extracellular space. But this offers no difficulty: the *concentration* of KCl in the interstitial space will be the same as that due to the larger amount that might freely diffuse throughout the whole muscle, since the space limitation for the KCl actually entering the muscle will exactly compensate for the relatively small amount so diffusing. Thus, the average degree of saturation of the extracellular space will be the same as that calculated for diffusion throughout the muscle.

In view of the above considerations, we may use Hill's graph (his fig. 5) to relate the diffusion constant, k, with the 0.90 average degree of saturation with 0.1 M KCl that corresponds to our 95% depolarization state. We find that $kt/b^2 = 0.86$, where $t =$ the time in minutes and $2b =$ the muscle thickness, which for our muscles had an average of about 0.07 cm. Hence, $k = 1.1 \times 10^{-4}$ cm^2/min. If the same procedure is used for determining the k values corresponding to 90% depolarization (at 6.5 min.), 85% (at 4.9 min.), and 75% (at 3.1 min.), then the respective k's are 0.85, 0.77, and 0.60×10^{-4} cm^2/min., which values represent a trend in variation of the calculated value of k that is to be expected (though details will be omitted) from the nature of the assumptions we have used. However, our value calculated from the state of the system corresponding to 95% depolarization undoubtedly is the most correct value and we may therefore conclude (at 20°C.) the diffusion constant of KCl in the muscle is of the order of 1.0×10^{-4} cm^2/min. Now this value is only about one-tenth of that for free diffusion of this substance in water at 20°C., which, according to Hill ('48), would be 10.3×10^{-4} cm^2/min. The much lower value we obtain, however, is to be expected, for in our experiments the KCl had to diffuse through various fascial sheaths, both external and internal, and furthermore, as already pointed out, the diffusion through the extracellular muscle space would be along irregular paths and not directly. These complications might very well account for the relatively low magnitude of k we calculate. At any rate, our value is quite comparable to that determined by Lorente de Nó ('50) for diffusion of KCl into the interfibrillar spaces of nerve (0.6 to 4.5×10^{-4} cm^2/

min.) and for NaCl into the same structure (0.68 to 0.8 × 10^{-4}), and by Hodgkin and Katz ('49) for diffusion of NaCl through the connective tissue sheath of the squid giant axon (0.7 × 10^{-4}). It is also of the same order as the diffusion constant for CO_2 in muscle at 22°C. (1.2 × 10^{-4}) as determined by Fenn ('28).

The close agreement among the values for the diffusion constant of KCl we determine, and those for KCl and NaCl in comparable systems just cited, indicates that our constant represents an accurate determination of the rate at which KCl diffuses into muscle. This, therefore, strongly suggests that the degree of depolarization of the muscle at any time depends solely on the speed at which the KCl has diffused to the fiber surfaces, and that, hence, the time required for depolarization itself by a given concentration of KCl acting at these surfaces is negligible. Now, it is conceivable that there might be a variety of mechanisms by which KCl actually causes the depolarization. Our diffusion analysis tells nothing as to the nature of such a mechanism, except that it involves some system at the fiber's surface that is very speedily alterable by changes in KCl concentration. Since this is in agreement with the mechanism of KCl depolarization expected in the Bernstein diffusion potential theory, our results, in this sense, must be considered as substantiating this theory. And, furthermore, we may therefore interpret the linear relation we and others have found in the usual Bernstein way. Hence, our results, in so far as they apply to the frog sartorius are in clear-cut contradiction to the conclusion of Lorente de Nó ('50, p. 238) "that Bernstein's and similar hypotheses on the nature of the membrane potential are inadequate."

It must be noted that the preceding analysis has been based on the depolarization kinetics of a fresh muscle exposed to 0.1 M KCl for the first time. The results graphed in figure 2, however, show that successive depolarizations of the same muscle by 0.1 M KCl become somewhat progressively slower, even though the final degree of depolarization in these trials remains essentially constant. I.e., previous treatment with KCl reduces the rate of depolarization in the later similar tests. The question then arises as to whether the decreasing rate of depolarization is due to a slowing of diffusion or to some interference in the speed with which the KCl causes a potential change at the fiber surfaces. It is difficult to see, however, why treatment with KCl should alter the system through which it diffuses in the muscle. Hence, the possibility exists that this salt may affect the actual depolarizability of the fiber membrane, although, if it does, its effects is not on the the fiber membrane, although, if it does, its effect is not on the speed with which this is attained. We will return to this point later in our discussion of the details of the action of RbCl.

We turn now to the rubidium effects. In confirmation of the work of other research (e.g., Feng and Liu, '49b) we have found that this ion causes a depolarization of the membrane which is not so pronounced as that due to potassium. Our own experiments deal only with 0.1 M RbCl and therefore do not permit us to determine any relation between Rb concentration and corresponding potential change. But Feng and Liu ('49b) have shown for nerve that the usual linear relation holds for Rb, and since this cannot depend on "the concentration ratio of rubidium inside and outside" they reject the Bernstein theory of resting potential. A somewhat similar argument is presented by Gallego and Lorente de Nó ('47). But this objection is not valid, for it misses the *essential* feature of the Bernstein hypothesis, i.e., that the membrane potential is a *diffusion* potential. In the normal cell the membrane polarization is assumed to be due to a potassium diffusion potential and its magnitude then varies linearly with the log Kin/Kout. If now the external KCl is replaced by RbCl, it is still possible to have another diffusion potential set up at the membrane, of the type referred to as a "chemical" diffusion potential, and its magnitude will vary somehow with the external concentration of Rb. Furthermore, if, following the original view of Bernstein, it is assumed that the nerve fiber membrane is not permeable to chloride and the intracellular anions, as has been demonstrated by Shanes ('48), and that (for generality) the mobilites of K^+ and Rb^+ in the membrane are, respectively U_K and U_{Rb}, then the Henderson equation for the potential is:

$$E = 58 \log \frac{U_K \, K_{in}}{U_{Rb} \, Rb_{out}} \text{ mv.} \tag{1}$$

This equation shows that the membrane potential will vary linearly with the log of the external Rb^+ concentration, as has in fact been found experimentally.

Gallego and Lorente de Nó ('47) also reject the Bernstein theory especially on the grounds of their experimental result that the Rb potential is easily reversible upon restoration of treated nerve to, e.g., Ringer's solution. This position is to us incomprehensible, for the reversibility of any diffusion potential, since it is a thermodynamic potential, is one of its outstanding features. Thus, in the sense of our above discussion, which is quite properly based on the generalization that the Bernstein hypothesis implies that membrane potentials are in essence diffusion potentials, both the linear relation and the reversibility of the Rb depolarization as found for nerve are not in contradiction to, but rather in support of this hypothesis.

Our own muscle results moreover, suggest that the depolarization by 0.1 M Rb involves a simple diffusion potential system. Let us consider the fact found by us that 0.1 M RbCl applied to the muscle surface causes an average potential drop of some 23 mv. We assume that, in the absence of the inevitable shunting in our whole muscle preparations, this drop would be double,[3] i.e., 46 mv. Taking the true resting membrane potential as 80 mv. (Ling and Gerard, '49), then the estimated value of the potential across the muscle fiber membrane, which is exposed externally to 0.1 M RbCl, is 80 — 46 = 34 mv., with the external solution positive in respect to the intracellular medium. To compare this with a diffusion potential theoretical value, we assume, to a first approximation, that at the muscle membrane a diffusion front is set up which is represented by the system (0.1 M) $K^+ + A^-$/(0.1 M) $Rb^+ + Cl^-$, where the left phase corresponds to the intracellular medium, and A^- symbolizes the internal anions. The Henderson equation for this system (noting that concentrations are the same inside and outside, and that the mobility of A^- is zero in the membrane) is:

---

[3] This factor seems reasonable, since our measurement of depolarization by 0.1 M KCl, which causes very nearly complete obliteration of the true resting membrane potential, is about 38 mv. But, the value of this potential, as directly measured by Ling and Gerard ('49) is of the order of 80 mv., i.e., about double what we measure.

$$E = 58 \frac{U_K - (U_{Rb} - U_{Cl})}{U_K - (U_{Rb} + U_{Cl})} \log \frac{U_K}{U_{Rb} + U_{Cl}} \text{ mv.} \qquad (2)$$

in which the U's represent the relevant mobilities of the ions in the membrane. If, following the work of Conway and Moore ('45), we set $U_K = 1$, $U_{Rb} = 0.33$ and $U_{Cl} = 1$, then $E = 37.5$ mv. Considering the many simplifying assumptions that have been made, this value predicted by equation 2 is close to that which we estimate for the membrane potential in this case; and this indicates that the Rb potential, not only in its linear relation, but also in its magnitude, is explainable simply as a diffusion potential.

Another feature of the action of Rb of great interest is the slowness of rate with which its depolarization is developed as compared to that of K. The reduced rate of the action of Rb cannot be explained by a difference in diffusion speed from the external medium to the fiber surfaces, for, if anything, this should be faster for Rb than for K since the former has, at least in H_2O, a somewhat greater diffusion constant. If, however, the Rb must actually penetrate into or through the membrane before its effect develops, then its rate of depolarization would be slowed by this penetration time. A similar action would not be involved for K, since, at least for the diffusion potential mechanism postulated for its action, only an approach to the outer surface of the membrane and not a penetration into it would suffice to change the membrane potential. These considerations may well account for the relative slowness with which the Rb depolarization builds up.

A final feature of the action of Rb is its depressive effect on the depolarizability of the membrane by K. Our results prove that for some hours after exposure of the muscle to 0.1 M RbCl, and the reversal of its effect by soaking in Ringer's solution, the action of 0.1 M KCl leads to a considerably smaller depolarization than is obtained before the Rb action. K does not act this way, for our experiments demonstrate that at least three successive actions of 0.1 M KCl, separated by intervening periods of soaking in Ringer's solution, always lead to essentially the same lowering of the potential, even though these equal final effects are attained at a somewhat slower pace in the later K actions.

We can only speculate as to the cause of the Rb depressive action. One possibility is that the Rb causes some relatively long lasting alteration of the colloidal structure of the membrane that interferes with its subsequent depolarization by K. It might be argued that K should have a similar effect, but this does not seem likely since the membrane is, normally, probably rather heavily saturated with the K ion and addition of extra external K would hardly introduce any new condition. But the presence of the external Rb, especially since it acts in the absence of the normally present Na and Ca, would radically alter the chemical environment of the membrane and thus possibly lead to our observed effect.

Thus, the present research strongly indicates that the various features we have studied of the effects of K and Rb on the resting potential of skeletal muscle are in general explicable within the framework of the Bernstein diffusion potential theory. In particular, our results concerning the kinetics of K-depolarization are consistent with this theory, and the discussion of our Rb results on muscle and those of others on nerve are also in harmony with it. Even though this theory may yet be shown to be inadequate, our analysis indicates that the claims of inadequacy put forward by Lorente de Nó and Feng and Liu are not supported by the facts. In addition to what has already been presented in this respect, we furthermore note that we take issue with Feng and Liu's view that the membrane potential cannot be explained as a diffusion potential since, as they note, K and Rb have similar effects on the resting polarization. We agree that some of these effects are similar, e.g., the linear relation; but this similarity, as we have shown, can be interpreted within the general meaning of the Bernstein theory. But our work shows that Rb has several actions that are different from those of K: the relative slowness of the kinetics of its depolarization, and its depressive effect on subsequent K-depolarizations. It is clear that these results invalidate any criticism of the Bernstein mechanism which is based on the similarity of the actions of these two ions. Nor is it necessary to infer that because their effects are in certain respects dissimilar, that the diffusion theory is wrong, for as we have discussed above, even these differences are explainable within the Bernstein hypothesis.

Our investigation finally proves that the various sheaths of the frog sartorius present no great impediment to the diffusion, certainly of K — and very likely of Rb also — in their movement through the muscle to the fiber surfaces. This is in opposition to the results of Feng and Liu which indicate that the epineurium of amphibian nerve does act as a diffusion barrier. But there may be no real contradiction here, since quite possible differences in chemical composition, e.g., in lipoid content, of the relevant sheaths could account for the opposite results. Nevertheless, in the work on nerve there is still left unresolved the contradiction between the following: Lorente de Nó's ('50) evaluation of the diffusion constant of KCl into sheathed nerve ($0.6 — 4.5 \times 10^{-4}$) which, at least at the lower limit, is close to that we determine for muscle and which therefore indicates quite rapid diffusion, and the extreme slowness of KCl depolarization of the intact nerve. It should be noted, however, that the relevant diffusion constants have been estimated by means of different physiological tests for penetration of the salt into the nerve. It is evident that procedures involving varying physiological signs of penetration might lead to different evaluations of a diffusion constant, and this demands, as indicated by Feng and Liu ('50), the need for direct chemical methods for the solution of this problem.

SUMMARY

1. Studies have been made of depolarization of the frog sartorius muscle by various concentrations of KCl and by 0.1 M RbCl. Particular attention has been given to the kinetics of depolarization by these substances, and to the effect of Rb on the subsequent action of K.

2. Except for very small concentrations, there is a linear relation between the external log K and the depolarization.

3. The kinetics of the depolarization by 0.1 M KCl is very rapid; and an analysis of this process shows that its time course is apparently dependent solely on the rate of diffusion of KCl through the muscle sheaths and interstitial spaces, with a diffusion constant of 1.0×10^{-4} cm^2/min. It is concluded that depolarization by KCl at the fiber surfaces is essentially instantaneous. As measured by rate of depolarization, the muscle sheaths, contrary to those of nerve, are not an effective barrier to diffusion.

4. 0.1 M RbCl causes a maximal depolarization which is only about 61% of that due to 0.1 M KCl; and it does so along a slower time course than is characteristic of KCl.

5. Treatment of the muscle with 0.1 M Rb Cl causes a very slowly reversible depression of the depolarizability of the fibers by subsequent KCl action.

6. These results are discussed in relation to the Bernstein diffusion potential theory of the membrane potential. The facts alone serve to refute various objections to this mechanism; and the inferences drawn from the facts are shown to be consistent with the theory.

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COMMENTS
AND
COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

THE IMPORTANCE OF SODIUM ION FOR FERTILIZATION

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It is frequently assumed that sodium ion is an inert component of physiological saline solutions and of sea water, serving only to maintain the osmotic and ionic equilibrium (Hoff, '50). It was shown over 50 years ago, however, that sodium ion is essential for rhythmic contraction of turtle ventricle (Greene, 1898; Lingle '00) and *Gonionemus* muscle (Loeb, '00) and for excitation of frog muscle and nerve (Overton ,'02); only lithium would substitute for sodium ion. Lillie ('32) on the other hand notes that sodium ion is not essential for activity of cilia or spermatozoa, or for the contraction of the *Vorticella* stalk, and concludes that it is not essential for excitation. Recently, the question has arisen again with the demonstration by Lorente de Nó ('47) and more recently

Hodgkin and Katz ('49) and Huxley and Stämpfli ('51) that sodium ion is intimately concerned with excitation and the action potential of nerve. The present note is concerned with the question whether sodium ion is essential to fertilization in the egg of *Strongylocentrotus purpuratus.*

Lorente de Nó ('47) has introduced the use of choline chloride as an indifferent electrolyte in isotonic solutions for bathing nerve fibers. We accordingly developed a formula for an artificial sea water in which all of the sodium ion is replaced by choline, the balance of the other ions in sea water remaining unchanged (table 1). This mixture will be referred to as choline sea water (CSW); in addition, ordinary sea water (SW) and an artificial sea water (ASW) of normal ionic composition were used.

TABLE 1

Composition of solutions used

SALT	CONCENTRATION, GM/LITER IN	
	Artificial sea water	Choline sea water
NaCl	22.9	....
Na_2SO_4	3.8	....
$NaHCO_3$	0.19	....
NaBr	0.082	....
Choline–Cl	....	63.0
KCl	0.70	0.40
KBr	....	0.10
$MgCl_2 \cdot 6H_2O$	9.4	3.9
$MgSO_4$	....	3.2
$CaCl_2 \cdot 2H_2O$	1.3	1.3
H_3BO_3	0.011	0.011
KOH	to pH 8.2	to pH 8.2

Ovaries were removed from specimens of *Strongylocentrotus purpuratus* collected the previous day and stored overnight in the refrigerator. The ovaries were placed in dishes of sea water and allowed to shed. The eggs were transferred to finger bowls of sea water, and thence into 4 experimental dishes containing respectively SW, ASW, CSW, and a mixture of equal parts of ASW and CSW. They were washed twice with the respective solutions to remove any SW carried over with the eggs, and then allowed to remain in the experimental solutions for varying periods in different experiments. A portion of the eggs was removed from each dish to a dish of sea water, and each of the 8 dishes was then inseminated with a small amount of "dry" sperm. After an interval the number of eggs showing fertilization membranes in each dish was counted as an index of the success of fertilization. The eggs were followed through the first cleavage, but no counts were made at this stage since a large number of irregular cleavages were noted in many cases.

TABLE 2

The activation of Strongylocentrotus eggs in solutions with and without sodium ion. (Significant deviations from sea water controls in bold face)

INITIAL SOLUTION	TRANSFERRED AFTER 45 MINUTES TO	PER CENT ACTIVATION (MEMBRANE ELEVATION)	χ^2
Sea water	Sea water	99	0
Artificial Sea water	Sea water	94	2.46
	Artificial sea water	**92**	6.05
Artificial sea water plus choline sea water	Sea water	99	0
	Artificial + choline	**90**	11.1
Choline sea water	Sea water	**83**	25.8
	Choline sea water	**0**	650

The results of one experiment are given in table 2. Four additional experiments with different batches of eggs gave substantially equivalent results. In every case, fertilization was substantially normal in SW or ASW, although frequently somewhat less in ASW than in SW. No signs of fertilization were observed in CSW. This could not be attributed to a ''toxic'' action of choline, since eggs kept in CSW for from 15 to 90 minutes fertilized normally when removed to SW; the percentage of eggs fertilized was less than with controls in SW, however. Fertilization was essentially normal in mixtures of ASW and CSW, though less effective than in the controls. Since these differences in percentage of eggs fertilized were evident in all the artificial sea waters, it seems unnecessary to attribute them to a specific effect of choline.

The failure of fertilization in CSW is attributed primarily to a failure of response in the egg. A sample of eggs was placed in CSW, washed and inseminated. The sperm remained vigorously active for a period of 2–3 minutes, during which time many spermatozoa made contact with eggs, without causing any response. After about three minutes, the activity of the spermatozoa ceased. Again the inactivation of the spermatozoa was not attributable to any toxic action of choline. Spermatozoa remained active in the ASW-CSW mixture for long periods, and the transfer of inseminated eggs from CSW to SW, or even the addition of a small amount of sodium ion (one drop of SW added to 10 ml of CSW) served to revive the spermatozoa caught in the egg jelly coat and to initiate the surface reactions leading to membrane elevation.

Cleavage was observed in all solutions except CSW, but eggs which were kept in CSW for 15 minutes or longer, transferred to SW and inseminated at once showed a very large percentage of abnormal cleavages indicative of polyspermy.

We may conclude from the foregoing results that sodium ion is essential for fertilization in *Strongylocentrotus,* and also for motility of the *Strongylocentrotus* spermatozoon. Taken with the results of others showing that sodium ion is essential for excitation of nerve, skeletal muscle, heart muscle, and the muscle of *Gonionemus* this conclusion suggests that sodium ion plays a fundamental role in excitation generally. The specific exceptions mentioned by Lillie ('32) should be reexamined. The exact nature of this role is uncertain, except for the observations that the action potential of nerve is accompanied by a rapid uptake of sodium ion.

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JOURNAL OF
CELLULAR AND COMPARATIVE PHYSIOLOGY

Vol. 38 OCTOBER 1951 No. 2

CONTENTS

Frances Green Kallman. Substrate specificities and inhibition of intracellular alkaline phosphatases associated with cytoplasmic granules sedimented by differential centrifugation. Five figures 137

Michael Ladd, Lawrence G. Raisz, Charles H. Crowder, Jr. and Lot B. Page. Filtration rate and water diuresis in the seal, Phoca vitulina. Two figures 157

Bodil Schmidt-Nielsen and Knut Schmidt-Nielsen. A complete account of the water metabolism in kangaroo rats and an experimental verification. Six figures 165

Leslie E. Edwards and Carolyn T. Edwards. A method for the in vitro collection of pure gastric juice from isolated gastric mucosa. Four figures 183

A. Van Harreveld. Asphyxial potassium loss of mammalian nerve 199

Lela Mae Jeffrey. The effect of pH of external solution on oxygen consumption of onion root tissue. Two figures 207

Edward L. Redford and Jack Myers. Some effects of ultraviolet radiations on the metabolism of Chlorella. Eleven figures 217

Aser Rothstein and Rebecca Meier. The relationship of the cell surface to metabolism. VI. The chemical nature of uranium-complexing groups of the cell surface. Four figures 245

Alexander Sandow and Harvey Mandel. Effects of potassium and rubidium on the resting potential of muscle. Three figures 271

Comments and Communications:

Bradley T. Scheer and Marlin Ann Ray Scheer. The importance of sodium ion for fertilization 293

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